

AN ABSTRACT OF THE THESIS OF

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Title: The Function and Structural Characteristics of Conserved
Regions Within *Escherichia coli* Small Subunit Ribosomal RNA

Abstract approved: _

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Ribosomes are multicomponent macromolecular particles and are essential for the survival of cells in all organisms. The ribosome's universal function is to catalyze polypeptide synthesis through translation of mRNA transcripts. Ribosomes from *Escherichia coli*, eubacterial organisms, have a sedimentation coefficient of 70S and are composed of 30S and 50S ribonucleoprotein subunits. The small ribosomal subunit is an assembly of 21 different proteins and a 16S ribosomal RNA. Within the 16S rRNA there are a few short stretches of universally conserved sequences spanning positions 517-533, 1394-1408, and 1492-1506. Clear functions for these sequence zones have not yet been assigned.

Here I report a kinetic analysis of these highly conserved regions in the 16S rRNA and within the 30S ribosomal subunits. Binding affinity was measured in experiments that were based on protection from nuclease S1 digestion of short oligodeoxynucleotides hybridized to the designated regions. DNAs hybridized to regions 1400 and 1500 show

significant differences in the apparent dissociation constants when measured in 30S particles as opposed to those found for 16S rRNA. Region 525 showed no difference in kinetic behavior.

To further elucidate the functional and structural role played by the region centered about C1400 in 16S rRNA, a four nucleotide deletion was constructed within this region. The deletion was introduced by direct RNA manipulation using DNA/RNA hybridization, RNase H digestions, and ligation of the correct RNA fragments with T4 RNA ligase. I improved ligation efficiency of large RNA molecules by including a connector looped short DNA oligomer. Recycling products through phenyl boronate agarose (PBA-30) column also improved the efficiency of ligation.

The mutagenized 16S rRNA fully reassembles into 30 particles and the altered 30S subunit possesses all of the normal ribosomal proteins. Altered ribosomes were functional in *in vitro* translation of MS2 mRNA. The altered ribosomes have lower translational activity relative to controls. Here I present indirect evidence suggesting that the decrease in the synthesis of MS2 coat proteins is the result of premature termination.

The altered 16S RNA in ribosomes had an apparent dissociation constants with DNA probes comparable to those found for normal 16S rRNA. This suggest that the RNA is less flexible in the particle relative to normal 30S subunits. The deletion at 1400 did not have any effect on the physical properties of the 1500 region, as measured by DNA hybridization. A minor, but significant, effect on the 525 region was observed. A possible RNA/RNA interaction within the 30S particle is proposed to account for this observation.

The Function and Structural Characteristics of
Conserved Regions Within *Escherichia Coli*
Small Subunit Ribosomal RNA

by

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DEDICATIONS

I would like to especially dedicate this thesis to my mother, my father, my brothers, my sisters, and my nieces and nephews. Dedication is extended to my wife's mother and father. I am very fortunate to have such wonderful people to encourage and support me to achieve my ambition.

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THE FUNCTION AND STRUCTURAL CHARACTERISTICS OF CONSERVED REGIONS WITHIN *ESCHERICHIA COLI* SMALL SUBUNIT RIBOSOMAL RNA

CHAPTER 1

INTRODUCTION

1.1. THE RIBOSOME

Ribosomes are large multicomponent complexes consisting of many proteins and several RNAs. They are the only cell organelle that is common to all living cells (Schachman *et al.*, 1952; Petermann and Hamilton, 1957; Tissieres and Watson, 1958), carrying out the many catalytic activities required to synthesize proteins (Littlefield *et al.*, 1955; Gierer, 1963). Ribosomes and their ribonucleic acid components are distinguished by their sedimentation coefficients expressed in Svedberg units (S). The structure of ribosomes is similar but not identical in all species.

Soon after the ribosome had been characterized as a site for protein synthesis *in vivo* (McQuillen *et al.*, 1959; Nomura *et al.*, 1984), it became apparent that ribosomes from bacteria can be distinguished from those of higher organisms on the basis of their sedimentation coefficients ('S' values). Since that time, prokaryotic ribosomes have been referred to as '70S' and those of eukaryotes as '80S'. Ribosomes are complexes consisting of two unequal size subunits. RNA molecule(s) together with proteins make up each subunit.

There are significant differences between prokaryotic ribosomes and those that are found in the cytoplasm of eukaryotic cells. Bacterial ribosomes consist of two major non-identical components, a small 30S and a large 50S subunit. The small subunit consists of 21 proteins and a 16S RNA (1542 nucleotides), while the large subunit has 34 ribosomal proteins (rproteins) and two non-identical RNAs, 23S rRNA and a small 5S rRNA (2904 nucleotides and 120 nucleotides, respectively), see Figure 1-1. Ribosomal proteins were named according to the position they migrate on two-dimensional polyacrylamide gels (Geyl *et al.*, 1981). Small subunit proteins are designated by the letter "S", while "L" refers to proteins derived from the large ribosomal subunit.

The proteins of 30S particle are S1 to S21 and in general the higher the number the lower molecular weight. Their molecular weights range from 25,800 to 8,300 Daltons (Geyl *et al.*, 1981). There is one copy of each of these protein molecules in the small ribosomal subunit. The 50S subunit contains 34 proteins (L1-L34). Protein L12 differs from L7 only by an acetyl group at the amino-terminus. Although the relative amounts of proteins L7 and L12 may vary depending on the bacterial growth rate, there are a total of four copies per 70S ribosome (Thammana *et al.*, 1973; Subramanian, 1975). Furthermore, proteins L26 and S20 are identical and present in the 70S ribosome as a single copy, presumably at the subunit interface (Hardy, 1975; Capel *et al.*, 1987) and the protein designated L8 is a complex of protein L10 with an L7/L12 tetramer (Pettersson *et al.*, 1976). Thus the bacterial ribosome contains 53 different proteins with 21 (including S20) in the small subunit and 32

Figure 1-1. A summary of the *E. coli* 70S ribosomal components. The overlapping two oval configurations are presented in the interest of simplicity. The proper structure is shown in Figure 1-5.

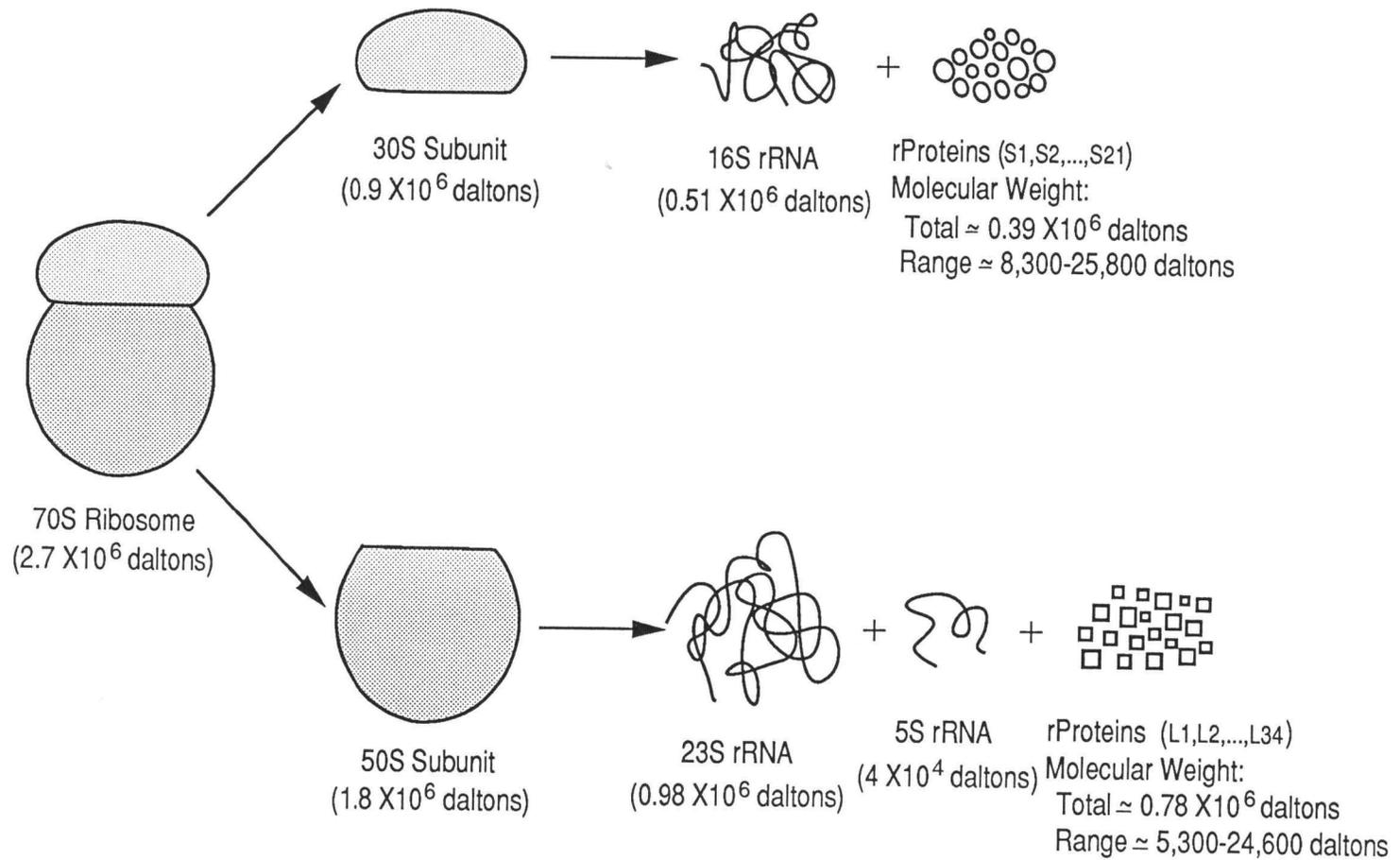


Figure 1-1

(excluding L26) in the large subunit.

Eukaryotic ribosomes are somewhat more complex. Cytoplasmic (80S) ribosomes are composed of a 40S and a 60S subunit. The small subunit consists of 18S rRNA (0.7×10^6 Daltons) plus about 30 proteins. The 60S subunit contains about 50 proteins and three rRNA species (28S, 1.7×10^6 Daltons, 5.8S, 5×10^4 Daltons, and 5S RNA, 4×10^4 Daltons). Chloroplast ribosomes (70S) resembles those of bacteria. Mitochondrial ribosomes, on the other hand, are heterogeneous; those in *Neurospora* (70S) are very similar to *E. coli* ribosomes whereas mammalian mitochondrial ribosomes (55S) lack 5S rRNAs and are unique in that they have a larger number of proteins (Wool, 1979).

In vitro reconstitution of bacterial ribosomal particles, pioneering work of Traub and Nomura (1968), made it possible to study the function of each ribosomal component. The assembly of the particles requires nothing more than its constituents, rRNAs and rproteins. A reconstituted particle has all the biological functions of natural particles (Traub and Nomura, 1969). The ease of *in vitro* assembly might be, at least partially, due to the basicity of the majority of the rproteins which probably accounts for their strong association with the RNA, which is acidic (Kurland, 1977). Nevertheless, rRNA/rprotein interaction is a highly specific one (Schaup *et al.*, 1970; 1972). The relative ease of total ribosomal reconstitution is an indication that the process of this complicated assembly as well as the information needed to form ribosomal quaternary structure reside fully in the primary sequences in the proteins and rRNAs

1.2. BIOLOGICAL ROLE OF RIBOSOMES

Ribosomes provide the spatial and structural framework for aligning the protein translational process. The mRNA is decoded in the 5' to 3' direction. Amino acids are transported and placed correctly in a growing polypeptide chain by the transfer RNA through proper tRNA/mRNA (codon/anticodon) alignment. Protein biosynthesis is a highly complex process involving, in addition to the multicomponent ribosome, amino acyl-tRNA synthetases and cytosolic protein factors. In prokaryotic cells mRNA is usually polycistronic, whereas all known eukaryotic cellular mRNA are monocistronic. Translation of mRNA transcript into a polypeptide progresses through three main phases: initiation, elongation, and termination.

1.2.1. Initiation of Translation

Cellular protein synthesis is aided by a number of specific RNA and protein interactions. The initiation phase of translation starts with 70S ribosome dissociation into its 30S and 50S subunits (Nomura *et al.*, 1984). The dissociation is stimulated by the protein factors IF-1 and IF-3. They act by shifting the equilibrium strongly towards dissociation as a result of their binding to 30S particles (Godefroy-Colburn *et al.*, 1975).

The ribosome binds to the initiator region at the 5'-end of mRNA, or 5'-proximal region in the case of polycistronic message, and moves in

a 5'-3' direction. The 30S binding to mRNA and selection of the correct initiation codon (in most cases AUG) involves a specific interaction with a sequence at the 3'-end of the 16S rRNA. A stretch of polypurine nucleotides varying between 3 and 9 preceding the initiator codon has been proposed by Shine and Dalgarno (1974) to base pair with a complementary sequence near the 3'-terminal of the 16S rRNA. The affinity of the bacterial ribosomes for the mRNAs containing the Shine-Dalgarno (SD) sequence is over an order of magnitude higher than that of mRNA lacking this sequence (Calogero *et al.*, 1988). The anti-SD sequence (5'-CACCUCCUU-3') is found in all eubacterial cells and, with minor variations, in archaeobacteria and chloroplasts (Dams *et al.*, 1988). In eukaryotic systems, in contrast, the 40S subunit binds to the 5'-terminal of the mRNA, containing a m⁷G cap structure, and scans through until it reaches the first AUG initiation codon (Kozak, 1987).

Once the small subunit is properly positioned at the initiation site, a special initiator tRNA that is formylated at the amino terminal of the methionine (fMet-tRNA_f^{Met}) binds to the AUG start codon within the "P" site of the 30S ribosomal subunit (Figure 1-2) (Gold *et al.*, 1981). This step requires the IF2 protein factor in association with a GTP. Binding of the 50S to the 30S pre-initiation complex triggers the release of all the initiation factors (IF1, IF2, and IF3) and hydrolysis of GTP. The GTP is in a bound complex to IF2. The formation of 70S initiation complex primes the ribosome to begin elongation phase.

In eukaryotes, protein synthesis is initiated by the binding of eIF2/GTP/Met-tRNA_i^{Met} ternary complex to the 40S subunit. This is

Figure 1-2. Illustrative scheme of the initiation step of translation in prokaryotic systems. The sequence 5'GGAGG3' is the Shine/Dalgarno site located about 5-10 bases upstream of the initiation codon.

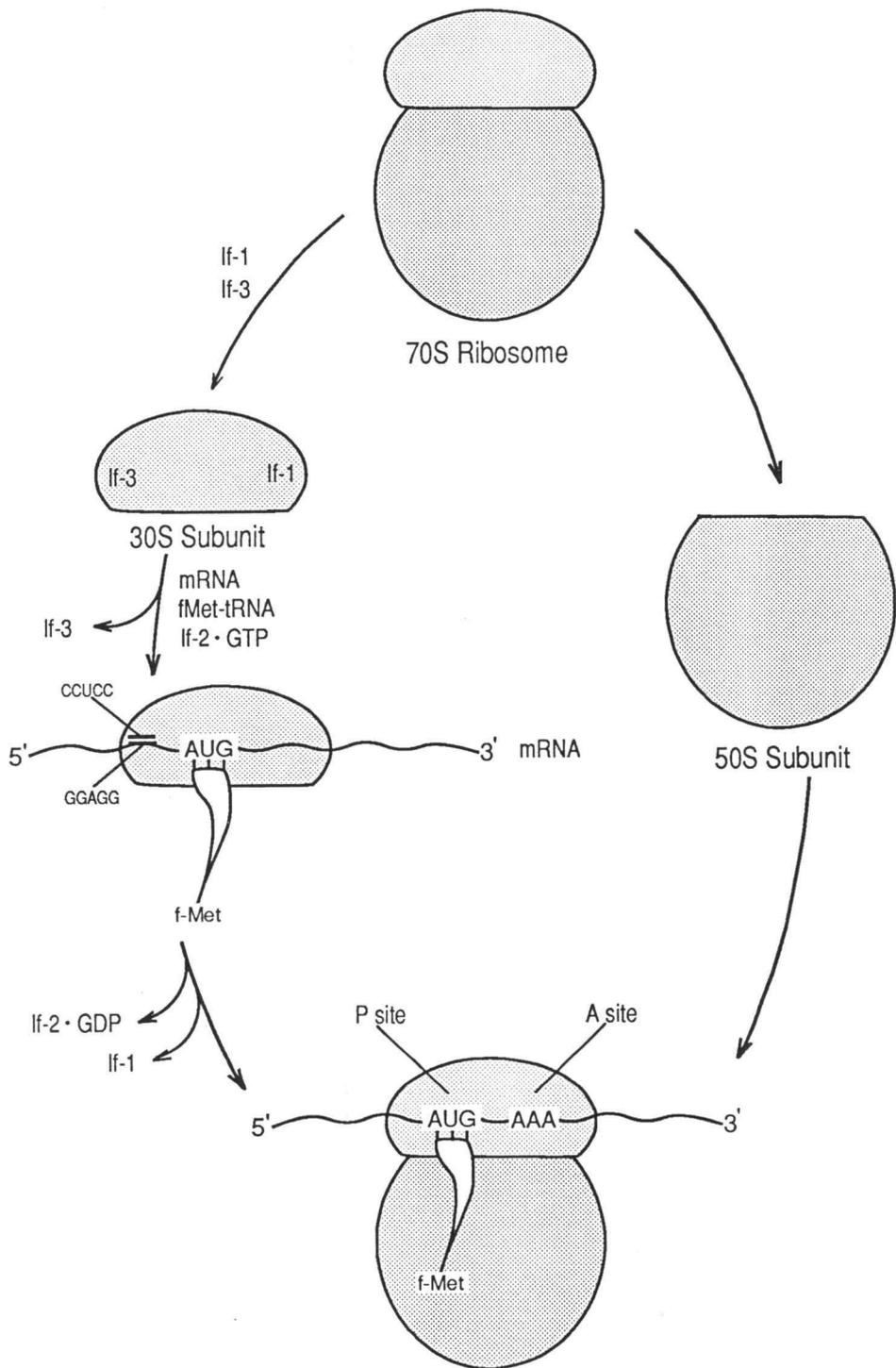


Figure 1-2

followed by mRNA association and an ATP-dependent movement of the 40S subunit along the message to the AUG initiation codon (Kozak, 1987). Eukaryotic cells require many more protein factors, eIf1, eIf2, eIf2B, eIf3, eIf4 (A,B,C,D,F), eIf5, and eIf6 to initiate protein synthesis. An additional difference is that initiator Met-tRNA is not formylated. It has been suggested that formylation is not strictly required for initiation at the first cistron of the polycistronic messages in prokaryotic systems, but it is essential for initiation at succeeding cistrons (Petersen *et al.*, 1984). This suggestion is based upon the finding that binding to 70S ribosomes inevitably requires the formylation of Met-tRNA_f^{Met}, whereas unformylated Met-tRNA_f^{Met} can bind to 30S ribosomal subunit (Petersen *et al.*, 1976). This could explain the lack of formylation in the eukaryotes, which have monocistronic messages.

1.2.2. Peptide Chain Elongation

Figure 1-3 outlines the steps involved in the elongation phase of translation processes. Initiator tRNAs bound to the ribosome reside at the so called "P" (Peptidyl) site. The other incoming aminoacylated tRNA's occupy the adjacent "A" site and they enter as a ternary AA-tRNA/EF-Tu/GTP complex. The amino acid (this amino acid is lysine in Figure 1-3) bound to its tRNA is brought into proper positioning by correct base pairing of mRNAs codon to the tRNAs anticodon. GTP hydrolysis allows the dissociation of the EF-Tu.GDP binary complex from the ribosome. A second elongation factor, EF-Ts, catalyzes the

Figure 1-3(A, B). Illustrative scheme showing peptide elongation step of protein synthesis in prokaryotes. Panel A shows the process of aminoacyl-tRNA binding to the A site of 70S ribosome. B shows the transpeptidation and the translocation steps.

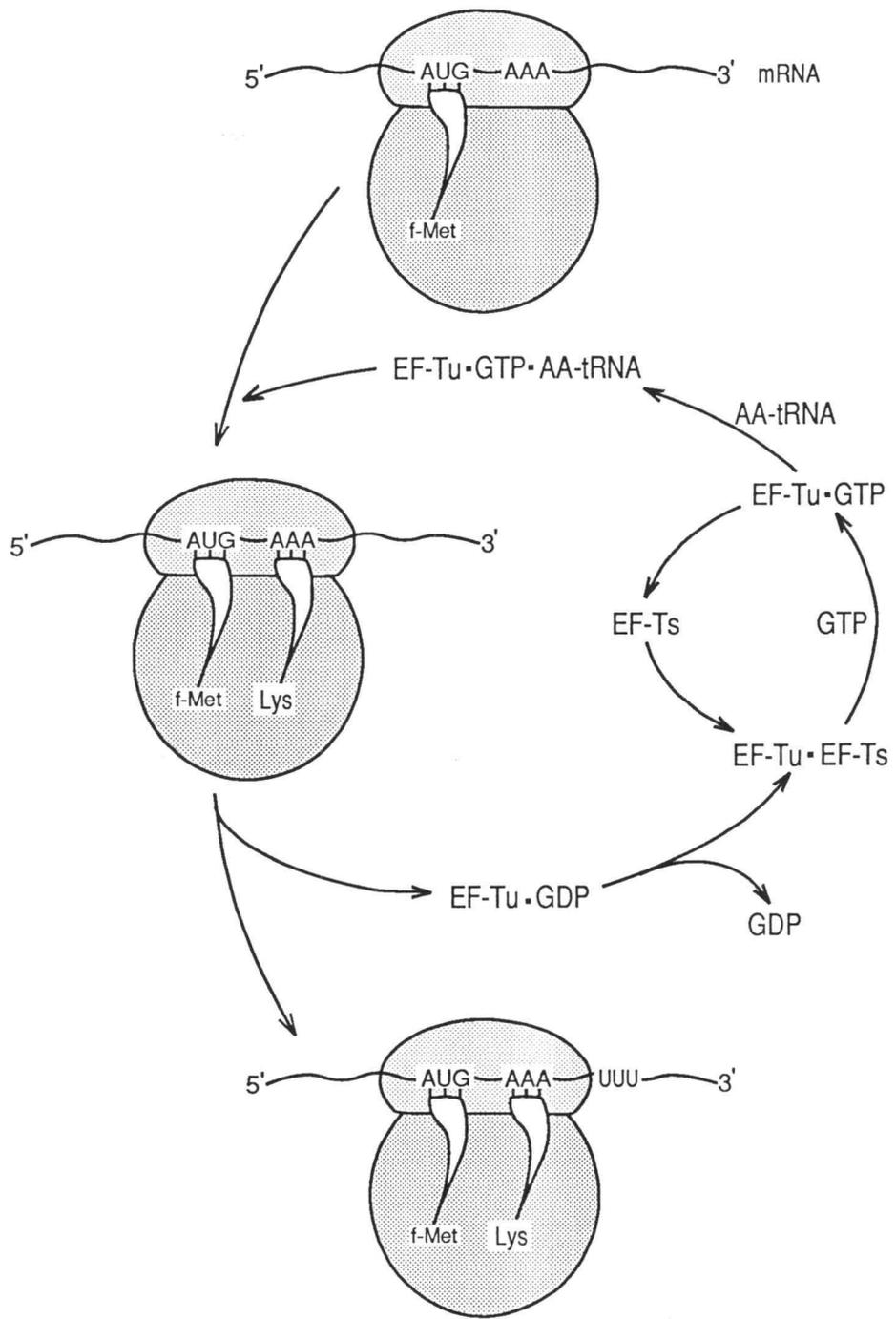


Figure 1-3A

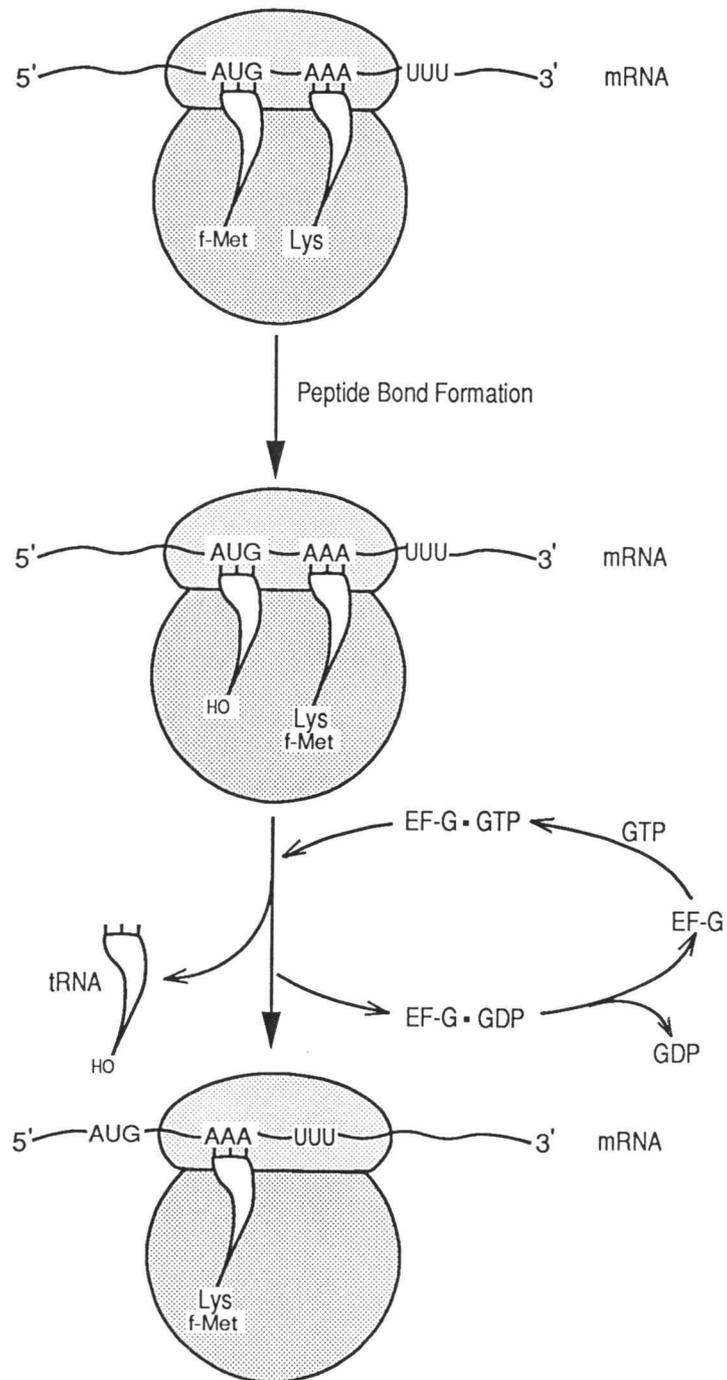


Figure 1-3B

regeneration of Ef-Tu.GTP to be able to interact with other aminoacyl-tRNAs to form the AA-tRNA.EF-Tu.GTP ternary complex (Kaziro, 1978).

Transpeptidation (peptide bond formation) occurs between the carboxyl group of the methionine and the amino group of the amino acid at the A site (i.e., lysine). The activity of the peptidyl transferase has been localized to the large ribosomal subunit (Monro, 1967; Maden *et al.*, 1968) presumably involving L7/L12 rprotein region (Traut *et al.*, 1986). Following peptide bond formation the dipeptidyl-tRNA is translocated from the A to the P site. This is mediated by the protein factor EF-G (Johnson *et al.*, 1982). The uncharged transfer RNA is released from the P site during this translocation process, possibly through the Exit site (E site) (Nierhaus and Rheinberger, 1984), accompanied by GTP hydrolysis. Comparable protein elongation factors in the eukaryotic systems are Ef-1a, Ef-1b and Ef-2 (Moldave, 1985). The cycle is repeated for the addition of each amino acid to the growing chain.

1.2.3. Termination of Protein Synthesis

Eventually, the termination of polypeptide chains is signaled by one of three special termination codons (nonsense codons), UAG, UAA, or UGA, in the mRNA transcript (Figure 1-4). In prokaryotes two proteins (release factors RF-1 and RF-2) have different nonsense codon specificity while a third factor (RF-3) stimulates their action (Caskey, 1980; Buckingham *et al.*, 1987). In eukaryotes, on the other hand, only one release factor is involved (Moldave, 1985). Binding of the release

Figure 1-4. Illustrative diagram outlining the termination step of protein synthesis. RF stands for release factor.

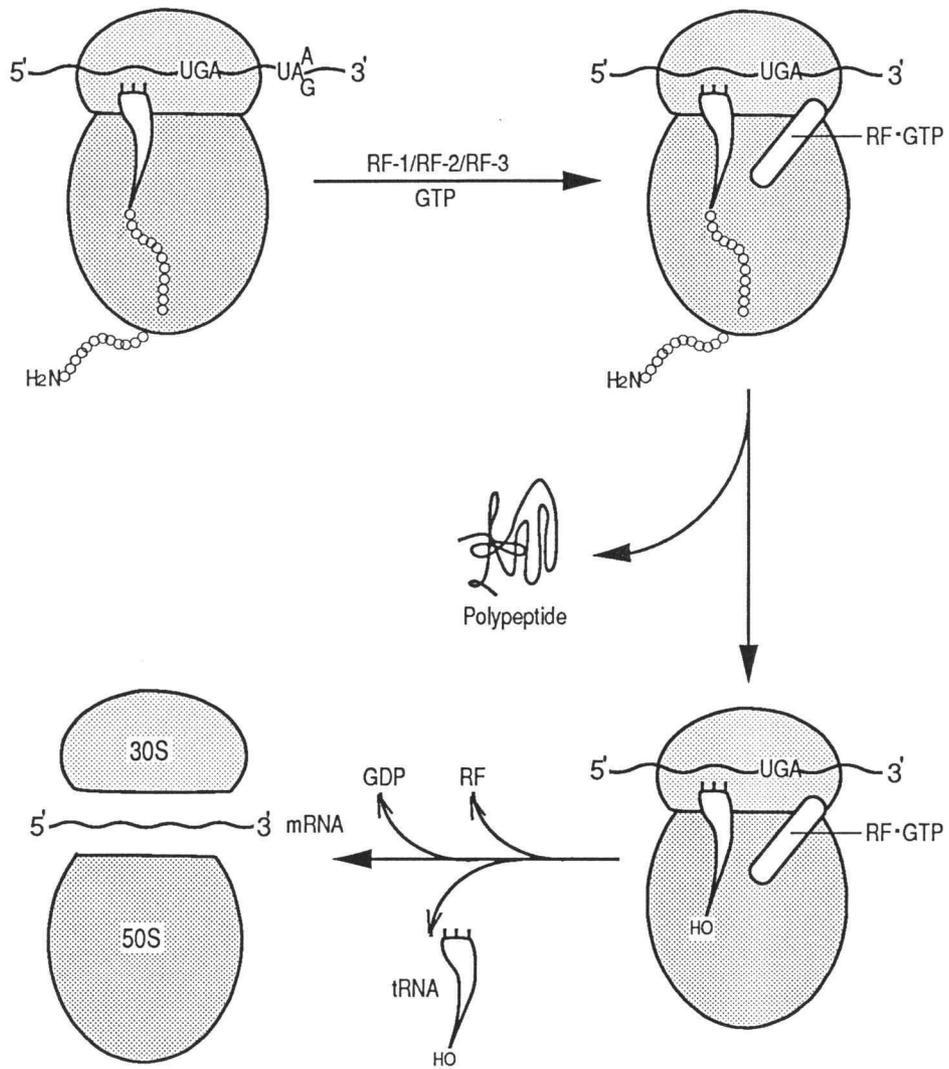


Figure 1-4

factors results in some sort of structural modification of the peptidyl transferase center of the ribosome. This leads to the hydrolysis of the peptidyl-tRNA rather than peptide bond formation (Caskey, 1980). Finally the polypeptide is released, the mRNA.Ribosome complex is dissociated and one cycle of protein synthesis is over.

1.3. PHYSICAL STRUCTURE OF RIBOSOMAL SUBUNITS

Ribosomal subunits lack any symmetry or regular or repetitive structure, but a considerable morphological resemblance exists between ribosomes of different organisms and cells (Wittmann, 1983; Verschoor and Frank, 1990; Wagenknecht *et al.*, 1989). In spite of the fact that ribosomes were first discovered in eukaryotic cells (Palade, 1955), most of the information available has come from studies on the prokaryotic ribosome. There is now a general agreement as to the shape of *E. coli* ribosomal particles (Figure 1-5). The small subunit is divided into main parts: the head and the base. A large lobe (platform) extends from the base and forms a cleft between it and the head (Stöffler and Stöffler-Meilicke, 1984; Lake, 1985). The general shape of the large subunit, like that of the small subunit, is asymmetric. It includes three projections extending from the main body. The one in the center is called the central protuberance. The two other side protuberances are termed the ridge and the L7/L12 stalk (left and right, respectively, when viewed from the interior side) (Oakes *et al.*, 1986).

Figure 1-5. Physical structure diagram of *E. coli* 70S ribosome and its 30S and 50S subunits. The sketch is a simplified drawing of what is seen with electron microscopy (see Lake, 1985).

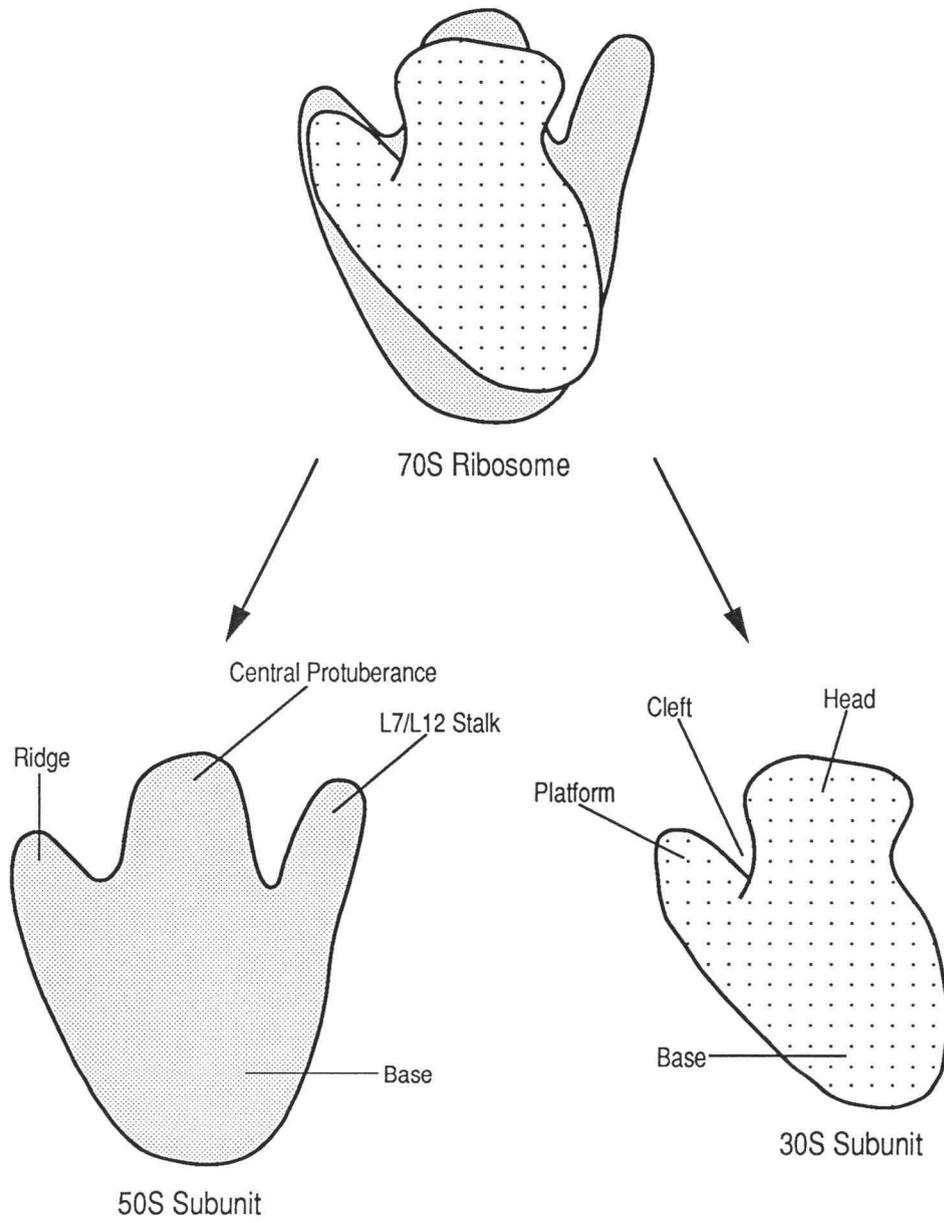


Figure 1-5

Current images of ribosomal subunits derive from three sources: electron microscopy, neutron scattering, and x-ray crystallography. Each of these methods has its own features but also its limitations. Although electron microscopy offers lower resolving power than the other two methods, it is performed under vacuum conditions of dehydrated ribosomes (Frank *et al.*, 1988; Boublik, 1990). Electron microscopy is most widely used for obtaining details regarding the structure of ribosomal particles (Lake, 1982; Wittmann, 1983; Korn *et al.*, Vasiliev *et al.*, 1983; Stöffler and Stöffler-Meilicke, 1984). However, the high background noises of the images has delayed the agreement on the proper three-dimensional model of 16S rRNA. X-ray crystallography allows the determination of structure at a higher resolution. But when dealing with large macromolecular assemblies such as ribosomes, obtaining sizable and perfect crystals is extremely difficult (Yonath and Wittmann, 1989; Trakhanov *et al.*, 1987). Neutron-scattering studies of ribosomal subunit, based on measuring the distance between pairs of rproteins, has provided a clear three-dimensional structure for *E. coli* 30S particles (Capel *et al.*, 1987). Neutron scattering of the large subunit of *E. coli* ribosome has been done by Nowotny *et al.* (1986), but the complete picture remains to be established. However, there is excellent agreement between the structure of *E. coli* 30S subunit obtained by neutron scattering and by immune electron microscopy (Stöffler-Meilicke and Stöffler, 1987; Capel *et al.*, 1987).

1.4. STRUCTURE OF 16S rRNA

1.4.1. Primary and Secondary Structure

Complete sequences of the large ribosomal RNAs were first reported for the *E. coli* 16S rRNA (Brosius *et al.*, 1980) and then for the *E. coli* 23S rRNA (Brosius *et al.*, 1981). Over 450 complete or at least 70% complete sequences of small subunit rRNA have NOW been published (Neefs *et al.*, 1991). About 270 sequences are those of eubacterial, 97 eukaryotic cytoplasmic, 19 archaeobacterial, and 44 mitochondrial small subunit rRNA. Some portions of the sequence of 16S rRNA show extensive variability, while certain regions display a high degree of conservation. There are a few short stretches of nucleotide sequence within the 16S rRNA that are universally conserved in all organisms, namely sequences spanning nucleotides 517-533, 1394-1408 and 1492-1506 of 16S rRNA (Woese *et al.*, 1983; Pace *et al.*, 1986). The sequence of the 1533-1542 zone is highly conserved in all prokaryotic cells (Gutell *et al.*, 1985), with the exception of *Giardia lamblia* (Sogin *et al.*, 1989), but found not to be conserved in eukaryotes. The true extent of structural conservation of rRNA becomes apparent when one comparing their secondary and tertiary structures.

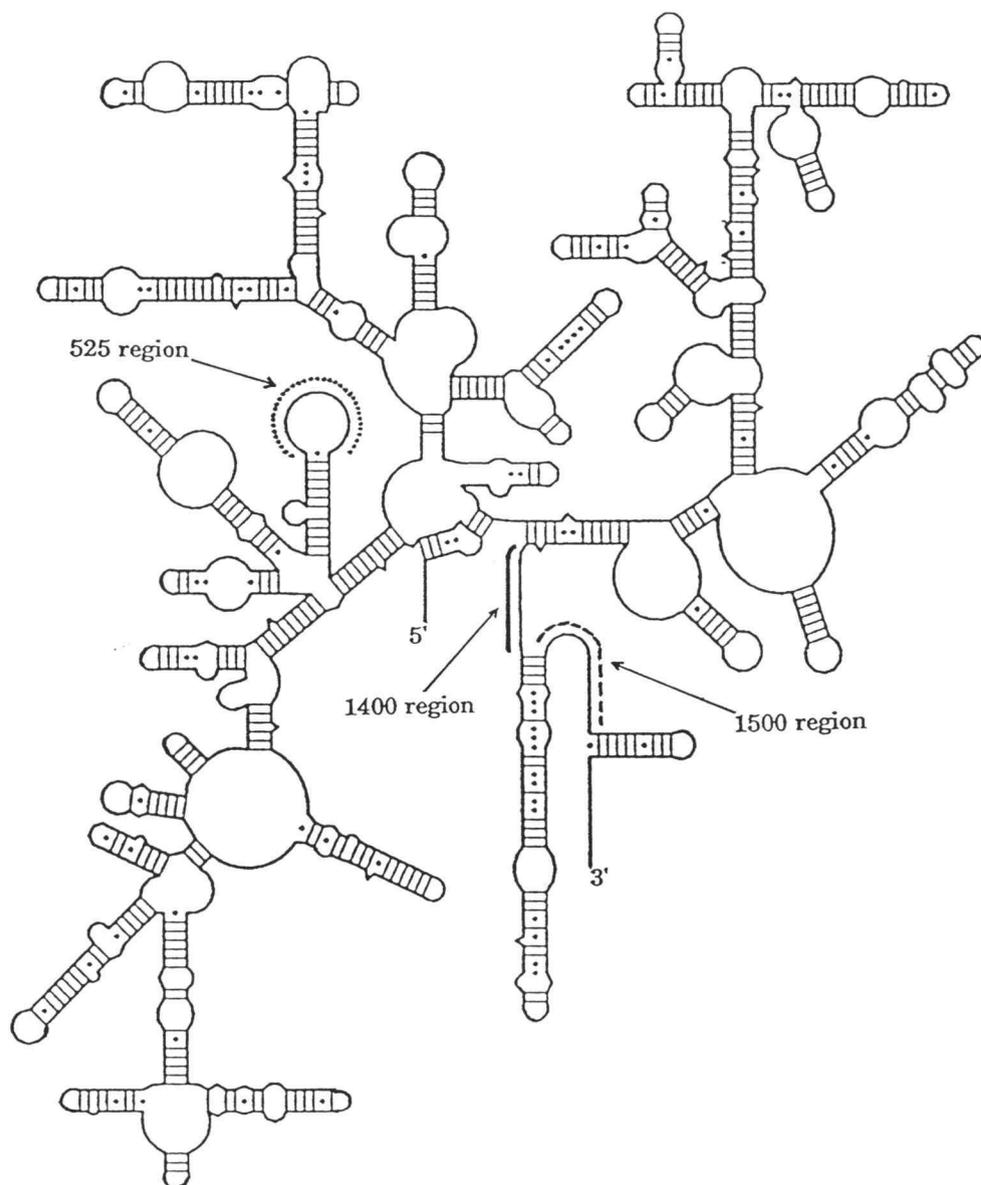
The first model for the secondary structure of the small subunit ribosomal RNA came from three independent research groups (Noller and Woese, 1981; Stiegler *et al.*, 1981; Glotz *et al.*, 1981). These models were found to be in excellent agreement with each other (Brimacombe

and Stiege, 1985; Gutell *et al.*, 1985) (Figure 1-6). In general, the *E. coli* 16S rRNA secondary structure is organized into three major domains plus a fourth minor domain involving the last 150 nucleotides at the 3'-end (Woese *et al.*, 1983; Gutell, 1985).

Two separate approaches have yielded a massive amount of data supporting the proposed 16S rRNA model. In one approach the RNA structure is studied through its susceptibility to chemical reagents and nucleases specific for either single-stranded or double-stranded RNA. Examples of chemical probes used for identifying the regions available for modification include diethylpyrocarbonate (A- and G-specific), kethoxal (G-specific), dimethyl sulfate (A- and C-specific) and bisulfite (C- and U-specific) (Noller, 1984; Woese *et al.*, 1983). The chemically modified regions are identified by reverse transcription of the modified regions (Moazed *et al.*, 1986). The movement of reverse transcriptase is blocked by certain chemically modified bases (Youvan and Hearst, 1979). Likewise ribonucleases, such as RNase A, T1 and T2, have strong preference for single-stranded regions and do not affect nucleotides masked by hydrogen bonding or involved in tertiary interaction (Glutz and Brimacombe, 1980; Stiegler *et al.*, 1981). In addition, cobra venom RNase was used to pinpoint double-stranded areas (Brimacombe, 1984). The same analysis has been applied to rRNA from several other organisms (Hogan *et al.*, 1984; Choi, 1985; Lempereur *et al.*, 1985; Rairkar *et al.*, 1988).

The theoretical approach using phylogenetic comparisons was first proposed by Fox and Woese in 1975. The highly conserved regions of

Figure 1-6. Secondary structure model of *E. coli* 16S rRNA (from Stern *et al.*, 1988). The underlined regions are the universally conserved sequences.



..... : 5' GCCAGCAGCCGCGGUAA 3' (525 region)
 ————— : 5' ACACACCGCCCGUCA 3' (1400 region)
 - - - - - : 5' AAGUCGUAACAAGGU 3' (1500 region)

Figure 1-6

the primary structure are used as a reference for aligning the secondary structure of related rRNAs from various organisms. It has been established that the secondary structure of the small subunit ribosomal RNA is conserved particularly with regard to base-paired regions (Woese *et al.*, 1983). If a base or bases change in part of a helix, it is compensated for by a complementary base change in the second strand of the same helix (Noller *et al.*, 1981; Woese *et al.*, 1983).

1.4.2. Tertiary Structure

The spatial arrangement of *E. coli* 16S rRNA is being investigated in several laboratories. Information regarding higher order structure of 16S rRNA comes from a combination of methods including immune electron microscopy (Stöffler-Meilicke and Stöffler, 1987), intra-RNA crosslinking (Brimacombe *et al.*, 1988; Schiiler and Brimacombe, 1988), DNA hybridization microscopy (Oakes *et al.*, 1990), neutron-scattering methods for measuring distances of protein component (Moore *et al.*, 1986), and protection against chemical and enzymatic probing (Stern *et al.*, 1988; Rahman and Schaup, 1990).

Packaging of 16S rRNA into the 30S particle involves significant structural rearrangements. Association of certain ribosomal proteins that are known to bind 16S rRNA in the initial assembly steps, such as S4, S8, and S15, increase the folding of the RNA (Mandiyani *et al.*, 1989). Ribosomal proteins S20, S17, and S7, on the other hand, are found to decrease the folding (Mandiyani *et al.*, 1989). Based on studies of

protection against chemical and enzymatic reagents, the groups of Noller and Woese group observed interactions between the 570 and 866 regions (Gutell *et al.*, 1986) and between the 510 and 530 pseudoknotlike loops (Woese and Gutell, 1989). Electron microscopic visualization of 30S subunits using biotin tagged DNA hybridization to localize certain regions of 16S rRNA on the surface of 30S subunit. Seven such zones have been observed: the 525, 700, 720, 800, 1400, 1500, and 1535 regions (Oakes *et al.*, 1986; Oakes and Lake, 1990; Olson *et al.*, 1988). The highly conserved regions of 16S rRNA, namely zones 525, 1400, 1500, and 1535, are found to be exposed and readily accessible on the ribosome surface (Rahman and Schaup, 1990).

Based on the data discussed above, three separate groups have proposed three dimensional models for *E. coli* 16S rRNA (Expert-Benzancon and Wollenzien, 1985; Brimacombe *et al.*, 1988; Stern *et al.*, 1988). The earliest model is of Expert-Benzancon and Wollenzien (1985). Based on intra-RNA cross-link results, they have used isolated 16S rRNA rather than intact 30S subunits as the substrate for the majority of the cross-linking studies. The model proposed by Brimacombe and co-workers is based on cross-linking studies (Brimacombe *et al.*, 1988; Schüler and Brimacombe, 1988) and supported by immunoelectron microscopic observations of positions of various specific nucleotides of intra-RNA crosslinks (Gonicki *et al.*, 1984) and of protein crosslinks (Stöffler-Meilicke and Stöffler, 1987) on the surface of the 30S ribosomal subunit. Moore's three-dimensional map of 30S rproteins (Moore, P.B.,

1988; Capel *et al.*, 1987) was used in building the 16S rRNA spatial model proposed by Stern *et al.* (1988).

1.5. FUNCTION OF 16S rRNA

1.5.1. 16S rRNA-mRNA Base-Pairing

The earliest well-established ribosomal inter-RNA interaction involves the 3'-end sequence (5'CCUCC3') of the 16S rRNA with its complement centered about 5 to 10 nucleotides upstream from the start codon in prokaryotic mRNA. The base-pairing was first proposed by Shine and Dalgarno (1975) and was thought to be necessary to set the correct reading frame at the initiation codon. Disruption of this interaction by removal of the anti-SD sequence causes a loss in the fidelity of translation *in vitro* (Abdul-Latif and Schaup, 1988). G to A transition at position 11 upstream of the AUG initiation codon in bacteriophage T7 mRNA abolishes translation of the 0.3 protein (Dunn *et al.*, 1978). Jacob *et al.* (1987) showed that C to U transition at position 1538 of 16S rRNA dramatically reduces *in vivo* ³⁵S-incorporation into many cellular proteins. Replacing the whole anti-Shine-Dalgarno sequence (replacing CCUCC with ACACA from position 1535 to 1539) caused the ribosome to no longer recognize natural mRNAs (Hui and DeBoer, 1987). Replacing the Shine-Dalgarno sequence in the message to complement the mutant ribosome restored translation and helped in selecting mRNA with altered complementary SD-sequence (Hui and De Boer, 1987).

The role of the anti-Shine-Dalgarno sequence extends beyond the initiation stage of translation. It has been suggested that SD/anti-SD base pairing might be involved in keeping the ribosome from slipping out of the correct reading frame (Dahlberg, 1989). This suggestion is based on the finding that a single nucleotide insertion between the SD sequence and the frame shift site in *E. coli* RF2 message decreases programmed frameshifting fivefold (Weiss *et al.*, 1988; Dahlberg, 1989). This mutation lowered the complementarity between the anti-SD region and a sequence on this mRNA essential for frameshifting. A transversion mutation within the anti-SD sequence restores complementarity and reestablishes wild type level of frameshifting. Therefore, the 3'-end region of 16S rRNA may act as a scanning machine that determines the proper reading frame by base pairing with internal Shine-Dalgarno sequences.

The molecular mechanism of translation termination is still obscure. There is some evidence suggesting base-pairing of the conserved tandem 5'-UCA-3' triplets at nucleotides 1199-1204 with the UGA termination codon (Dahlberg, 1989). C to U transitions and C1054 deletion within this 16S triplets causes a decrease in the efficiency of termination at UGA stop codons (Murgola *et al.*, 1988). There are no data concerning UAA and UAG termination codon interactions. However, a conserved UUA triplet at nucleotides 1211-1213 (Neefs *et al.*, 1991), which is complementary to either UAA or UAG stop codons, might be involved in a base-pairing termination mechanism (Raué *et al.*, 1990). Furthermore, C726 to G mutation in 16S rRNA resulted in

suppression of a UGA stop codon (Prescott and Dahlberg, 1990) and altered binding affinity for mRNA (Prescott and Göringer, 1990).

1.5.2. Initiation Factors Binding Sites

To comprehend fully the mechanism of ribosome function requires an understanding of the interactions of rRNA and protein factors. IF1, on one hand, is found to protect nucleotides G529 and G530 in the 525 loop and nucleotides A1492 and A1493 in the 1500 region from chemical attack (Noller, 1991). Since these same bases are found to be involved with A site conformation (Moazed and Noller, 1990), it is suggested that IF1 binds to 30S particles during initiation. Thus, blocking fMet-tRNA^{met} binding to the A site (Noller, 1991) allows the P site to become favored for the initiator tRNA attachment. Binding of IF3 to the 30S particle, on the other hand, shields some of the nucleotides that are part of regions found to be associated with the P site (700, 790, and 1500 regions) surrounding the decoding site (Muralikrishna and Wickstrom, 1989; Ehresmann *et al.*, 1986; Moazed and Noller, 1990). Furthermore, G to A transition at nucleotide 790 decreases IF3 binding to 30S particles (Tappich *et al.*, 1989). Based on these observations, it is suggested that the binding of IF3 at or near the decoding site creates an unfavorable non-specific tRNA interaction that causes conformational changes within the P site of the 30S subunit (Noller, 1991; Stern *et al.*, 1988). IF3 is thought to be involved in subunit dissociation because IF3 binding regions have been connected to subunit association (Brow and

Noller, 1983; Herr *et al.*, 1979). Finally, IF2 does not interact directly with the rRNA; its interaction might be through ribosomal proteins instead.

1.5.3. tRNA Interaction Sites

Interaction of tRNA with 16S rRNA at the "A-site" and "P-site" of the 30S subunit involves mainly the three universally conserved regions 530, 1400, and 1500. Moazed and Noller (1986, 1990) showed that tRNA protects nucleotides G529, G530, U531, G1405, A1408, A1492, A1493, and G1494 in the ribosomal A-site, while nucleotides A532, C1399, C1400, and G1401 were protected against chemical and enzymatic attacks in the P-site. In addition, certain conserved bases such as G693, A794, and C795 were protected in the ribosomal P-site (Moazed and Noller, 1990). These results were based on the observation that P-site related tRNA binding was fully puromycin reactive. A-site binding was accomplished by first filling the P-site with deacylated tRNA and then introducing the tRNA enzymatically into the A-site as an AA-tRNA/Ef-Tu/GTP complex (Moazed *et al.*, 1986). Earlier Prince *et al.* (1982) cross-linked cytidine at position 1400 of *E. coli* 16S rRNA to the wobble base tRNAs bound in the ribosomal P-site. Similar cross-links were observed in other prokaryotic and eukaryotic organisms (Ehresmann *et al.*, 1984; Ciesolka *et al.*, 1985). Chemical modification of many of these bases enables 30S subunits to participate in protein synthesis (Noller and Chaires, 1972; Noller, 1991). The same protection is observed when, instead of the whole tRNA molecule, only 15 nucleotides of the anticodon stem-loop are used (Rose *et*

al., 1983). This indicates that the anticodon stem-loop is the main contact site to 30S subunit.

A number of different aminoglycoside antibiotics, which are known to affect translation at the elongation stage (Moazed and Noller, 1987) are found to react with 16S rRNA (De Stasio *et al.*, 1989). Methylation of residues G1405 and A1408, by a methylase isolated from a gentamicin-producing organism, confer resistance to gentamicin and kanamycin (Beauclerk and Cundliffe, 1987). Paromomycin resistance is generated by a C to G transversion at 1409 in yeast mitochondrial 16S rRNA (Li *et al.*, 1982). Streptomycin resistance, on the other hand, is associated with mutational changes of residue 912 in chloroplast 16S rRNA (Montandon *et al.*, 1985; Etzold *et al.*, 1987) and in *E. coli* (Montandon *et al.*, 1986). Binding of streptomycin to 70S ribosomes or 30S subunits protects residues 911-915 from chemical attack (Moazed and Noller, 1987). The other streptomycin interaction site is found at position 523 in the small subunit rRNA of *E. coli* (Melancon *et al.*, 1988) and of chloroplasts (Gauthier *et al.*, 1988).

1.5.4. Intra-RNA Interactions

Two primary approaches are used to obtain information regarding 16S rRNA intramolecular interactions. One method is termed subunit fragmentation; where an intact ribosomal subunit is digested with ribonuclease and the protected RNA duplexes are separated and analyzed electrophoretically (Spitnik-Elson and Elson,

1979). Intra-RNA crosslinking is a more direct approach for revealing parts of RNA molecules that are close to each other. Both methods reveal information regarding helix regions in the secondary structure form, but they also provide data on interactions of RNA regions that are outside the secondary structure to provide insight on tertiary folding.

Ribosome fragmentation revealed several RNA-RNA contacts including 61-69/810-82, 417-429/722-732, 420-428/1330-1339, 858-864/1376-1383, 1025-1031/1178-1184, 1124-1130/1315-1320, and 1139-1144/1301-1307 (Spitnik-Elson *et al.*, 1985). These interactions are between RNA regions that are single stranded and conserved phylogenetically (Woese *et al.*, 1983; Gutell *et al.*, 1985). RNA cross-links are introduced using psoralen derivatives (Thompson and Hearst, 1983; Wollenzien *et al.*, 1979), direct u.v. irradiation (Stiege *et al.*, 1983), or nitrogen mustard (Stiege *et al.*, 1982). Cross-linked products were first identified by electron microscopy where the visibility of large loops indicated of intra-RNA contacts (Wollenzien and Cantor, 1982). DNA hybridization and subsequent digestion with nucleases (Hui and Cantor, 1985; Stiege *et al.*, 1988) was used later to analyze cross-linked products. Brimacombe *et al.* (1988) observed crosslinks at G31/A306, A246/C893, A694/A794, A1092/C1162, and U1126/C1281, whereas Cantor *et al.* (1986) found two possible duplexes of UGA923/UCA1534 or AUU921/GAU1532.

Evidence for specific conformational changes during different phases of ribosome function is beginning to accumulate. One such stage where the rRNA might undergo a certain conformational switch is during the translocation process. The entire mRNA-tRNA-peptide

complex must be physically moved by a distance of one codon (Glotz and Brimacombe, 1980; Thompson and Hearst, 1983). Recently Spirin *et al.* (1987), based on neutron scattering studies, showed that the ribosome has a different conformation in the post-translocation state as opposed to the pre-translocation state. They conclude that translocation is accompanied by a spatial displacement of some parts of the ribosome. Earlier Gupta *et al.* (1971) observed a slightly faster sedimentation profile for the pretranslocation ribosomal complex compared to the initiation complex. They suggested that the shape or compactness of the ribosome undergoes cyclic changes during the translation process. Psoralen cross-linking of ribosomal subunits indicates base pair switches in an active subunit compared to that of an inactive ribosomal particles (Ericson and Wollenzien, 1989). Base-pairing of regions 921-923/1532-1534 is seen in the active 30S subunit, whereas 923-925/1391-1393 base-pairing is observed in an inactive particles. Based on computer screened base complementary sequences, Kössel *et al.* (1990) suggested a possibility of 5'/3' terminus helix formation of bases 14-18/1530-1534 during the elongation phase. The 30S subunit conformation switches back to the proposed secondary structure conformation (Woese *et al.*, 1983) at the initiation step of protein synthesis. Furthermore, Leclerc and Brakier-Gingras (1990) proposed (based on identifying matching complementary sequence) a helix formation between residues 12-16 and 911-915. This alternate conformation requires the disruption of the 17-19/916-918 helix of the classical 16S rRNA secondary structure.

1.5.5. New Approaches

A promising way to study rRNA function is to use rRNA molecules with artificially altered sequences of suspected important regions, such as the highly conserved zones. The altered sequences can be produced either by site-directed mutagenesis of a gene or by direct RNA manipulation *in vitro*. An example of the former is provided by the work of several research groups who investigated the effect of deletions or substitutions of certain nucleotides within phylogenetically conserved regions of 16S rRNA. The effects of such modifications on cell growth (Jemiolo *et al.*, 1985; Thomas *et al.*, 1988), *in vitro* dipeptide formation (Denman *et al.*, 1989), and *in vivo* protein synthesis (Hui *et al.*, 1988) has been reported. This approach is limited either by the presence of background wildtype ribosomes or by the lack of sensitive functional assays. In addition, *in vitro* transcription of a mutagenized gene generates products that lack post-transcriptional modifications necessary for the full function of the mature rRNA transcripts.

The studies reported in this dissertation use a technique developed for direct *in vitro* deletion of specific nucleotides within the highly conserved 1400 region of the *E. coli* small subunit ribosomal RNA. With this method, T4 RNA ligase is used to ligate cleaved 16S rRNA fragments. One of the challenges of this study was to develop a strategy for enhancing the efficiency of such ligation reactions for large RNA molecules like 16S rRNA. Chapter 3 of this thesis describes the strategies used for direct manipulation of 16S rRNA, strategies used to

improve ligation, reconstitution of the altered 16S rRNA into ribosomal particles, and *in vitro* functional analysis of the activity of the altered ribosomes.

Sequence conservation against evolutionary pressure implies an important role for the universally conserved sequences of 16S rRNA in protein synthesis. In Chapter 2, I report a physical study of 30S ribosomal subunits. The study describes the kinetic characteristics of the highly conserved regions through their availability for interactions with other ribosomal or nonribosomal components. The method was based on hybridization of short synthetic oligodeoxynucleotides to a complementary region of interest and subsequent protection of the hybrid DNA from single-stranded-specific nuclease digestion. Apparent dissociation constants of DNA probes for regions 525, 1400, and 1500 of 16S rRNA were examined. The effects of removing four nucleotides from the conserved 1400 region on the physical properties of the 30S subunit and *in vitro* translation studies are reported and were correlated to a possible intra-RNA interaction between a sequence in the 1400 region with its complement within the 525 region.

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CHAPTER 2**PROBING THE FUNCTION OF CONSERVED RNA
STRUCTURES IN THE 30S SUBUNIT OF
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2.1. ABSTRACT

Ribosomes play an active role in protein biosynthesis. They provide a structural framework within which proper positioning of tRNA/mRNA takes place. Our understanding of some ribosomal processes that support these interactions is beginning to emerge. Ribosomal RNA conformation in ribosomal subunits, intramolecular interactions between different rRNA sequences within the confinement of the particles, and intermolecular interactions are presumed necessary to support efficient and accurate protein synthesis. Here we report an analysis of the disposition of 16S rRNA conserved zones centered about positions 525, 1400, and 1500 in the molecule. These areas were investigated in 30S subunits by using complementary oligodeoxyribonucleotides in conjunction with nuclease S1 digestion. All of the sequences examined in 30S subunits are accessible to DNA probes of 9 to 12 nucleotide residues in length. However, the kinetic characteristics of the respective DNA interactions with 30S particles vary significantly. By itself, the G + C content of the areas studied does not account for the different binding features. In addition to the investigation of normal 30S particles, a four base deletion within the 1400 region of 16S rRNA was analyzed. The alteration produces a significant change in the disposition of 16S rRNA in 30S subunits, suggesting a reduction in the freedom of movement of the altered zone in the 16S rRNA in the particle. In a factor-dependent *in vitro* protein synthesis system primed with MS2 mRNA using 30S subunits that have altered 16S rRNA, we found an

approximate 50% decrease in phage coat protein synthesis. The reduction could be due to a decrease in the rate of translation or premature termination of translation. We present evidence here, based on isotopic studies, which supports the later possibility. Premature termination may be due to disruption of possible base pairing between the altered conserved sequence zone and a short complementary region within the conserved zone centered about position 525 in 16S rRNA.

2.2. INTRODUCTION

Ribosomes have a central role in protein synthesis and display great complexity in terms of the number of macromolecular components present in the particles. There is considerable evidence that structural changes take place within the ribosome while it is active in protein biosynthesis (Dahlberg, 1989; Raué *et al.*, 1988). One may view ribosomal RNAs as structural cores for ribosomal subunits (Spirin *et al.*, 1979; Stuhrmann *et al.*, 1978). There have been many attempts to find direct correlations between higher order ribosome structure and the disposition of ribosomal components during the various phases of protein synthesis (reviewed in Thompson and Hearst, 1983; Brimacombe and Stiege, 1985). It came as no surprise when it was found that secondary and tertiary structural changes occur in rRNAs during active translation of mRNA (Brimacombe and Stiege, 1985). The dynamics of these changes is of particular interest with regard to several sequence zones within small subunit rRNA. The zones of interest are single-stranded and highly conserved among prokaryotes as well as eukaryotes. Several of these zones appear to be associated with codon-anticodon (decoding site) interactions (Moazed and Noller, 1986; Thomas *et al.*, 1988), while other areas are involved in direct interactions with mRNA (Shine and Dalgarno, 1975; Dahlberg, 1989; Sprengart *et al.*, 1990). Here we report investigations directed at ascertaining the accessibility of three of these highly conserved sequences in small subunit rRNAs. We

used *E. coli* for this work and the conserved sequences of interest center about positions 525, 1400, and 1500 in small subunit 16S rRNA.

Intense efforts have been undertaken to address the function of the RNA components in ribosomes, but little has been achieved in terms of linking specific RNA conformations to functions. At the moment, x-ray diffraction is the method of choice for determining the three dimensional conformation of large macromolecular complexes. Unfortunately, until crystallization and phase problems have been solved for 70S ribosomes, and its subunits, indirect biochemical approaches are the only alternative. Recent use of DNA hybridization techniques in conjunction with RNA-crosslinking (Cantor *et al.*, 1986) and ribonuclease-H digestion (Hill *et al.*, 1990; Skripkin *et al.*, 1982) have produced useful information with respect to the higher order structure of ribosomal RNAs. We have used DNA hybridization in conjunction with nuclease S1 digestion as a technique to map the disposition of sequence zones in 16S rRNA (Rahman and Schaup, 1990). Here we have extended those investigations to explore 30S conformational dynamics with regard to highly conserved small ribosomal subunit rRNA sequences. The local structure of these zones in 30S particles was examined by using zone specific DNA probe hybridization and protection of the hybridized DNA from digestion by S1 nuclease. Hybridization and digestion were accomplished by using conditions comparable to those employed in *in vitro* protein synthetic systems. DNA probe binding stoichiometry and K_{ds} in free 16S rRNA are significantly different from those found in 30S particles. Reconstituted 30S particles bearing a modified 16S rRNA

(missing 4 bases between positions 1400 to 1405) were also examined. The alteration affects DNA probe interactions with the distal 525 region as well as the modified site. In an *in vitro* protein synthesis system, it appears that the alteration causes premature termination, possibly affecting the stability of ribosomes during the elongation phase of translation.

2.3. MATERIALS AND METHODS

2.3.1. General Preparation

Salt washed or tight couple *E. coli* D10 ribosomal subunits were prepared as previously described (Hardy *et al.*, 1969; Noll *et al.*, 1973; Robertson and Wintermeyer, 1981). 30S particles were isolated from a 15-30% sucrose gradient following zone ultracentrifugation at 43,000 xg for 16 hr. Initiation factors (IF1, IF2, and IF3) and the high-speed supernatant, S100, were prepared as described by Hershey *et al.* (1981). Protein concentrations were determined using the Bio-Rad protein assay system with bovine serum albumin as the standard (Sigma). Cytidine-3',5'-[5'-³²P]bisphosphate (³²pCp) was made by using 3'-CMP and γ [³²P]-ATP (7000 mCi/mmol, ICN Radiochemicals) following the protocol of England *et al.* (1980). The formation of pCp was confirmed and quantitated by thin layer chromatography. Ribosomal RNA was prepared from phenol extraction of cell lysate and isolated by zone ultracentrifugation (Schaup *et al.*, 1970).

2.3.2. Preparation and 5'-end Labeling of Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were synthesized by the OSU Central Service Laboratory using an Applied Biosystem DNA synthesizer (Caruthers, 1985). Oligodeoxyribonucleotides were purified either by

high pressure liquid chromatography (Haupt and Pingoud, 1983) or electrophoretically by using a 20% acrylamide/7M urea gel (Ogden and Adams, 1987). This was followed by passage of the DNA through a Sep-Pak C18 column (Waters Associates) as described previously (Sanchez-Preschador and Urdea, 1984). Polynucleotide kinase from bacteriophage T4 (BRL) was used to label the 5'-terminus of oligodeoxyribonucleotides with γ [^{32}P]ATP according to Sambrook *et al.* (1989). Labeled DNA was purified by gel electrophoresis as described above and typically had a specific activity of 2.5×10^7 cpm/ μg .

2.3.3. Hybridization and S1 Nuclease Digestion

Specific 5'-labeled DNA was annealed to rRNA or 30S particles by using conditions adapted from previously described procedures (Sharp *et al.*, 1980; Belfort *et al.*, 1985; Rahman and Schaup, 1990). Following hybridization at 37°C in 20 μl hybridization buffer (40 mM Pipes, 400 mM NaCl, 3 mM MgCl₂, pH 6.8), the reaction mixture was diluted with 30 μl S1 nuclease digestion buffer (30 mM sodium acetate, pH 4.6, 280 mM NaCl, 4.5 mM zinc sulfate, 5% glycerol) containing S1 nuclease (Boehringer) at 5u/ μl final concentration. The digestion period was 45 min at 30°C. The digestion was terminated by the addition of 25 ml of 2.5 M ammonium acetate/50 mM EDTA (Sharp *et al.*, 1980). The products of the digestion were precipitated twice with cold ethanol with unlabeled tRNA (0.33 mg/ml) as carrier. The final precipitate was dissolved in electrophoresis buffer and the S1-resistant DNAs were resolved on 7 M

urea/20% polyacrylamide gels (Ogden and Adams, 1987) and visualized by autoradiography. DNA bands in the gel were removed and quantitated by using liquid scintillation spectrometry.

2.3.4. Preparation of Modified 16S rRNA

Modified 16S ribosomal RNA with a deletion within the 1400 conserved region was prepared by RNA digestion of DNA/RNA hybrids with RNase H as described in the accompanying report (Almehdi *et al.*, 1991). Removal of four bases was accomplished by using two sets of hybridization/digestion experiments with a shift of few bases in one of the hybrids. Digested RNA fragments were gel purified and the 3'-fragments were 3'-end labeled with [³²P]pCp and T4 RNA ligase (Meyhack *et al.*, 1978). The small 3'-fragment was ligated to the shortened larger 5'-fragment. Ligated RNA was run through a phenyl boronate column (Pace and Pace, 1980) to eliminate nonligated fragments. Deletion was confirmed by using nuclease S1 and a synthetic DNA complementing to the altered 16S rRNA construct (Almehdi *et al.*, 1991).

2.3.5. Ribosomal Proteins Preparation, *in vitro* Reconstitution, and Translation

Small subunit ribosomal protein components were isolated by acetic acid extraction of 30S subunits as described by Hardy *et al.* (1969).

Ribosomes were reconstituted by modification of the method of Traub *et al.* (Traub *et al.*, 1971; Abdul-Latif and Schaup, 1988). MS2 RNA-directed *in vitro* protein synthesis was measured by using either [¹⁴C] or [³H] labeled lysine and phenylalanine (NEN, 450 Ci/mol and 25 Ci/mmol respectively). The incorporation assay contained amino acids (2 nMoles each except for those added as label), 17 µg tRNA, 1 mM ATP, 0.12 mM GTP, 5 mM phosphoenolpyruvate, 0.1 µg pyruvate kinase in 40 mM Tris-acetate, pH 7.6, with 5 mM magnesium acetate, 50 mM KCl, 12 mM NH₄Cl, and 6 mM β-mercaptoethanol (Sugiyama *et al.*, 1969). The reaction mixture also contained purified initiation factors (0.3 µg IF1, 3 µg IF2, 0.6 µg IF3), 70 µg S100 proteins, and ribosomal particles (Van Dieijen *et al.*, 1975). The translation assays were initiated by the addition of 8 µg MS2 RNA (Boehringer), bringing the final reaction volume to 0.1 ml. The samples were incubated at 37°C for 30 min. Total incorporation of radioactivity was analyzed by trichloroacetic acid precipitation according to Mans and Novelli (1961). Some samples were further analyzed by SDS-polyacrylamide gel electrophoresis (Goldman, 1982). Radioactive materials were extracted from the gel and quantitated by liquid scintillation counting.

2.4. RESULTS

2.4.1. Oligonucleotides Binding to 16S and 30S

Table 2-1 shows the sequences of the DNA probes employed and the location of their complementary sequences on the *E. coli* 16S rRNA. Binding of these DNA probes to 16S rRNA is specific (Rahman and Schaup, 1990). Binding of the del-1396 DNA probe was assayed by using altered 16S rRNA (Fig. 2-1) and 30S ribosomal subunits. The other DNA probes were tested only with 30S modified and normal particles. Their specific binding to 16S RNA has been reported previously (Rahman and Schaup, 1990). Hybridizations were performed at 37°C with 5'-end labeled DNA, as described in Materials and Methods. High concentrations of nuclease S1 (single stranded specific nuclease) were used to assure the complete digestion of nonhybridized DNA. The only DNAs that escape degradation are those that are fully hybridized to the rRNA sites. Neither 16S rRNA nor 30S ribosomal particles were found to protect from S1 nuclease a DNA probe complementary to a sequence known to be in a double stranded portion of 16S rRNA (Rahman & Schaup, 1990).

Digestion products from experiments using the DNA probes shown in Table 2-1 were separated by polyacrylamide/urea gel electrophoresis. Fig. 2-2 (A, B) shows the protection of probes 1396 and del-1396. Here fixed amounts of 30S ribosomal subunits were titrated against increasing levels of DNA. These experiments were also done with DNA

TABLE 2-1
Synthetic DNA Probes

DNA	Position ^a	Length	Sequence ^b
1396	1396-1404	9	GGGCGGTGT
del-1396	1396-1408	9	TGACGGTGT
1493	1493-1504	12	CTTGTTACGACT
520	520-530	11	CCGCGGCTGCT

^a Position indicates the location on *E. coli* 16S rRNA of the complementary sequence.

^b shows DNA sequence in 5'-3' direction.

Figure 2-1. Partial secondary structural diagram of 16S rRNA. The diagram shows the 1400 region, and the site where the deletion was made is indicated by the offset sequence.

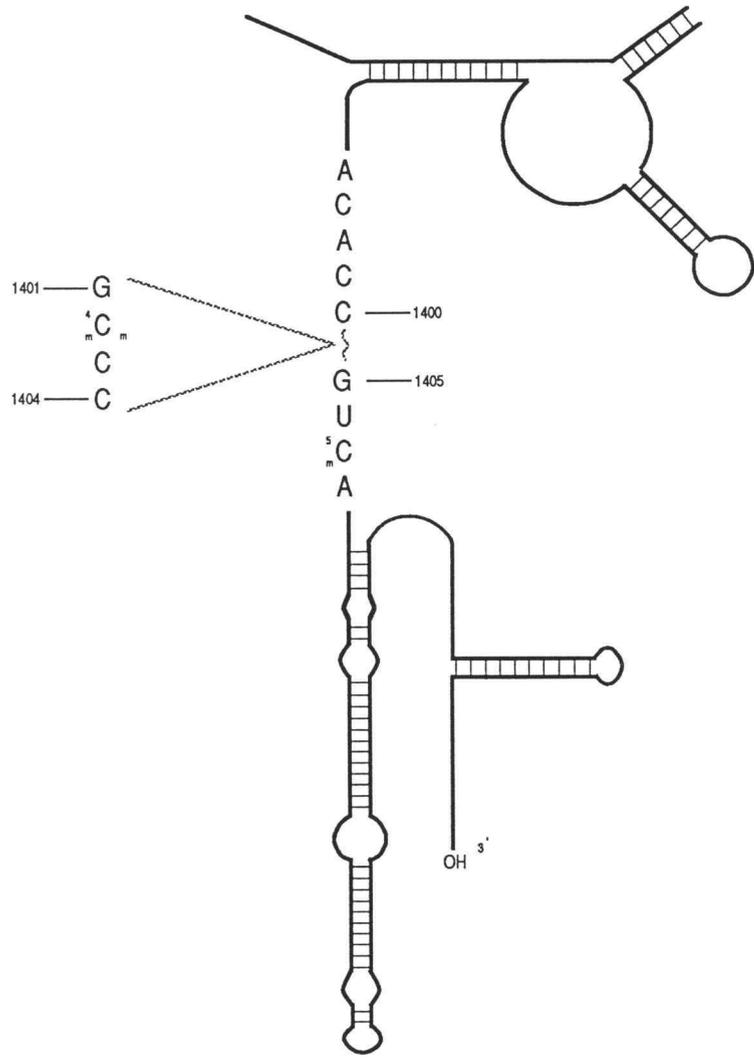


Figure 2-1

Figure 2-2. Autoradiogram showing protection against nuclease S1 digestion of 5' [^{32}P] labeled DNA probe hybridized to 16S rRNA in 30S ribosomal subunits. The numbers indicate the molar ratio of DNA to a fixed 1.5×10^{-13} moles of 16S rRNA. M shows intact DNA probe. C refers to the control reaction lacking RNA substrate. Binding of DNA 1396 to normal 30S ribosomes (Panel A) was compared with the binding of del-1396 DNA to altered 30S ribosomes (Panel B) as described in Materials and Methods. The denaturing polyacrylamide gel was 20% (26 x 24 x 0.08 cm) and run at 25 mA for 150 min at room temperature.

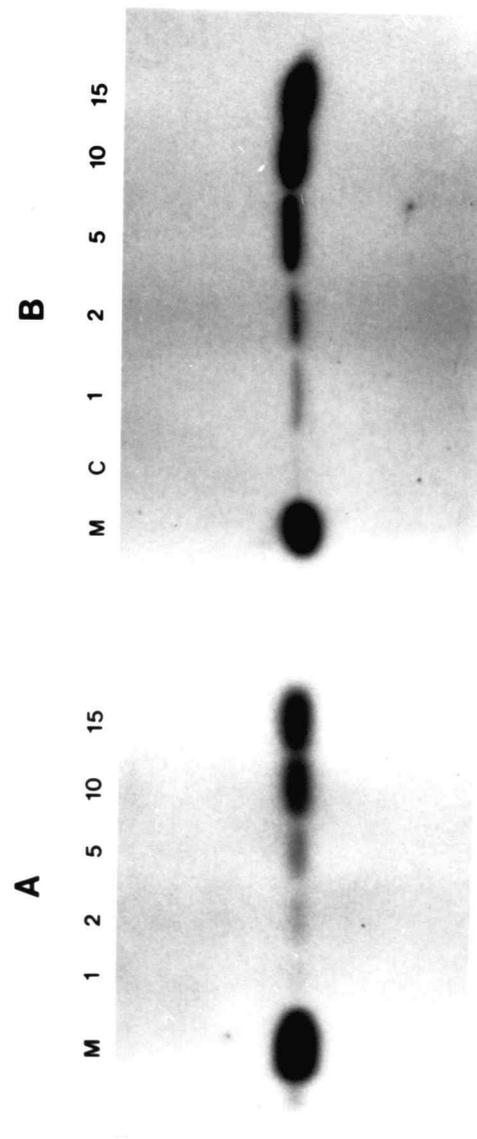


Figure 2-2 (A,B)

probes for zones 525 and 1493. Binding/saturation results were used to generate Scatchard plots (Scatchard, 1949). The results with modified 30S and normal 30S are respectively shown in Fig. 2-3 (A, B). These data were used to determine values for the apparent dissociation constants (K_d) for the hybridizations (Table 2-2). Ordinate intercepts yield the number of binding sites, n (van Holde, 1985) (Table 2-3). The value of n for 30S binding suggests that these sites are less accessible in 30S relative to free 16S rRNA where they bind stoichiometrically (Rahman & Schaup, 1990). However, the altered 30S has a significantly different K_d from the normal 30S. Altered 16S and the normal 16S rRNA show similar values of K_d (12.9×10^{-9} M and 13.2×10^{-9} M). Both the normal 16S and the altered RNA showed a 1:1 stoichiometry for complex formation.

2.4.2. Reconstitution and *in vitro* Translation Assay

Altered 16S rRNA has four ribonucleotides removed between positions 1400 and 1405 (Fig. 2-1). Based upon protein composition and sedimentation profiles, this manipulated 16S RNA assembled into 30S ribosomal subunits with the same efficiency as the normal 16S rRNA (data shown in the accompanying report; Almehdi *et al.*, 1991). To determine the functional properties of modified 30S particles, an *in vitro* protein synthesis system programmed with MS2 mRNA was employed. The MS2 phage-encoded peptides were labeled with [3 H]-Lys or [3 H]-Phe. [14 C]-Phe and [14 C]-Lys were used with normal ribosomes to provide a

Figure 2-3. Scatchard plot showing binding of DNA probes to 16S rRNA in 30S particles. Panel A: DNA 1396 binding to normal 30S particles. Panel B: DNA del-1396 binding to altered 30S ribosomes. Error estimation was based on linear regression analysis ($R^2 = 0.96$). The zones containing DNA in polyacrylamide gels, comparable to those shown in Figure 2-2, were excised from the gel and quantitated by liquid scintillation spectrometry. The number of moles of DNA in the protected bands were calculated by using the specific activity of the respective probes.

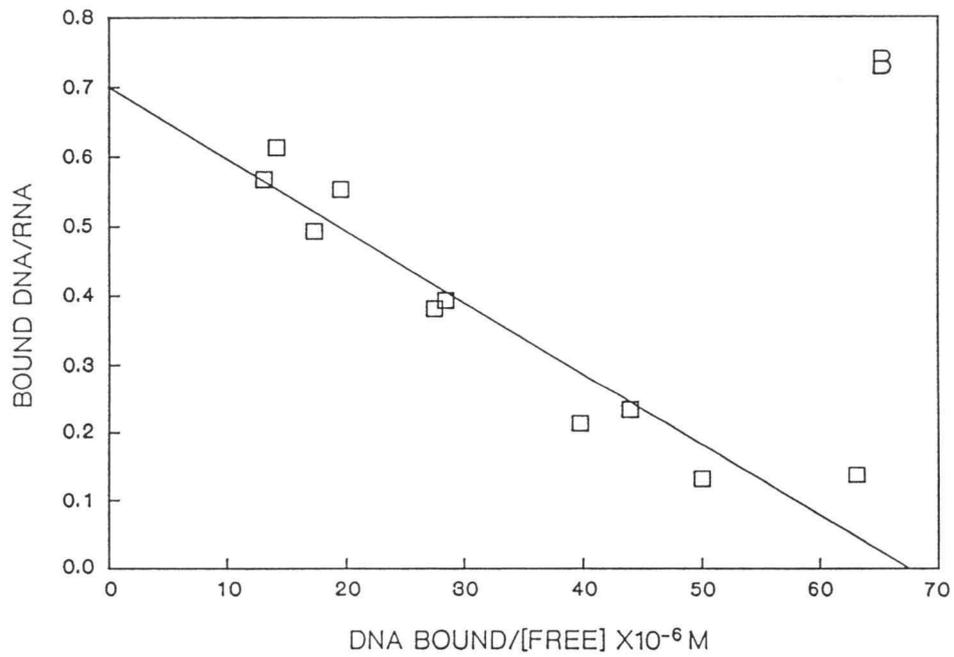
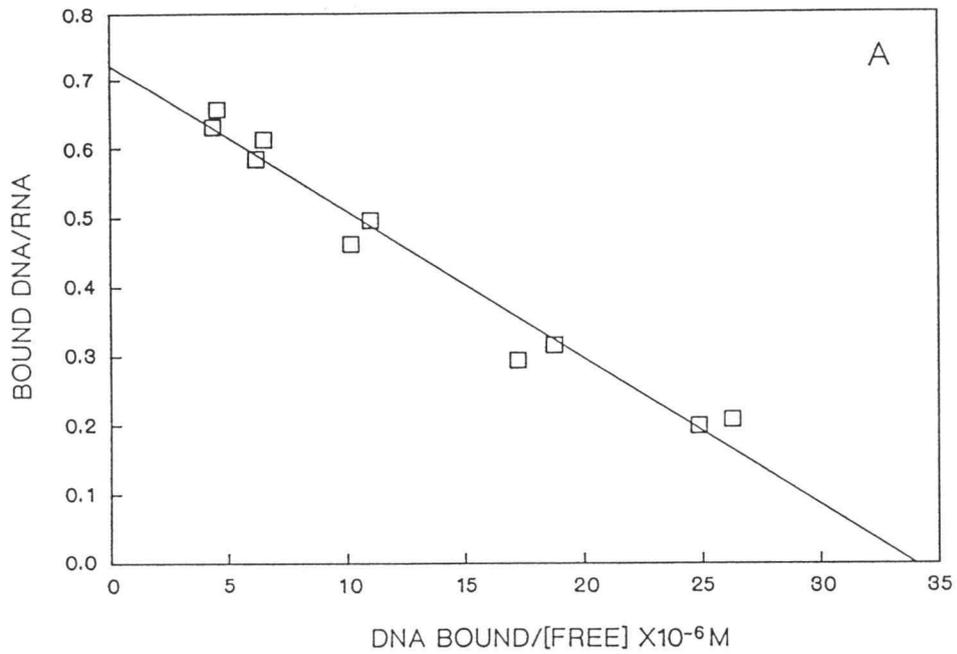


Figure 2-3 (A, B)

Table 2-2

Apparent Dissociation Constants (K_d) of DNA Binding to rRNA^a

Position of 16S	Normal 16S	Normal 30S	Altered 30S
1400 Region	13.3x10 ⁻⁹ M (± 0.6)	21.6x10 ⁻⁹ M (± 1.4)	12.1x10 ⁻⁹ M (± 0.8)
1500 Region	28.8x10 ⁻⁹ M (± 3.6)	12.8x10 ⁻⁹ M (± 1.0)	14.0x10 ⁻⁹ M (± 1.6)
525 Region	23.8x10 ⁻⁹ M (± 1.6)	20.7x10 ⁻⁹ M (± 1.2)	17.8x10 ⁻⁹ M (± 1.1)

^a Variation is ± one standard error x 10⁻⁹.

TABLE 2-3Binding Stoichiometry of DNAs Complementary to 16S rRNA^{a,b}

DNA	DNA/Normal 30S	DNA/Alter. 30S
1396	0.72 (± 0.03)	--
del-1396	--	0.74 (± 0.03)
1493	0.74 (± 0.03)	0.67 (± 0.04)
525	0.72 (± 0.03)	0.74 (± 0.03)

^a Values are expressed as molar ratios.^b Error expressed as standard errors.

standard. Incorporation of the radioactivity into hot trichloroacetic acid insoluble materials was a measure of overall polypeptide synthesis. When [³H]-Phe was used, the modified ribosomes displayed only about a 15% reduction in incorporation, but when [³H]-Lys was used the incorporation was reduced to about 50% relative to normal ribosomes. This apparent discrepancy is reconciled in the Discussion section. On polyacryl-amide gels the products of these translations in the presence of [³H]-Lys appear as a single peak migrating as expected for MS2 coat protein (Fig. 2-4). Both altered and normal ribosomes evidently produced the same product, but it is evident that the efficiency with which the altered ribosomes synthesize coat protein is substantially lower (<50%) than that of normal ribosomes.

Figure 2-4. In vitro translation of bacteriophage MS2 transcripts. [³H]-Lys-labeled products were analyzed by polyacrylamide gel electrophoresis and the distribution of radioactivity in the gel was measured as described in Materials and Methods. Electrophoresis was from left (cathode) to right (anode) and the products of [³H]-Lys incorporation using altered ribosomes (-□-) was compared to that of normal ribosomes (-■-). The graph shows an overlay of label distribution from two lanes on the same slab gel. The gel was 10% SDS-polyacrylamide (26 x 24 x 0.08 cm) and run at 150mA for 4.5 hours at 22°C.

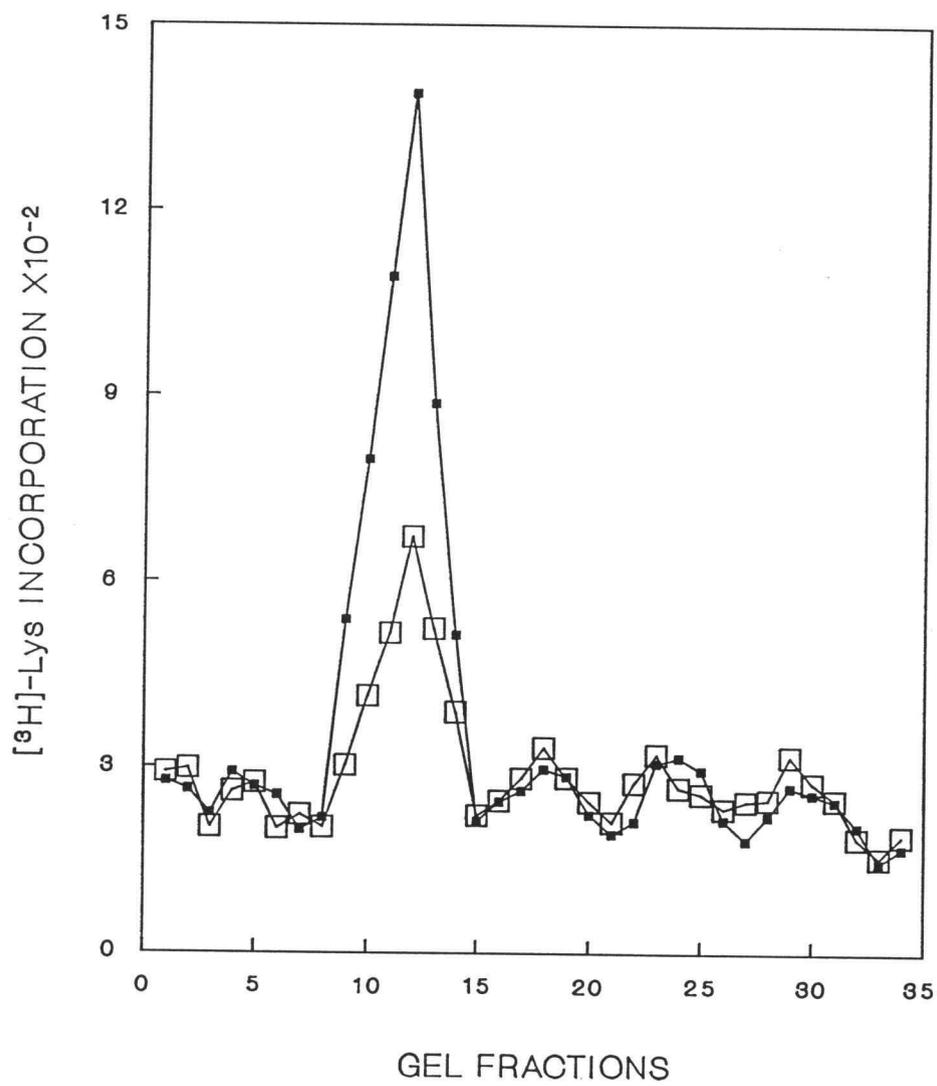


Figure 2-4

2.5. DISCUSSION

Three of the highly conserved regions in small subunit rRNA at positions 525, 1400, and 1500 in *E. coli* are largely single stranded in 16S rRNA and in 16S rRNA in 30S particles (Woese *et al.*, 1983; Noller, 1984; Rahman and Schaup, 1990). These zones are thought to form part of the decoding site where tRNAs interact with ribosomes (Moazed and Noller, 1986; Prince *et al.*, 1982). It is of interest to note that electron microscopic analysis of ribosome DNA hybrids suggests that these sequence zones are near the surface of the 30S subunit (Oakes *et al.*, 1986, 1990) and in positions precluding involvement in 50S subunit association (Meier and Wagner, 1985; Noller, 1984). The topological positions of these zones suggest that the changes we observe in probe binding characteristics are not clearly a consequence of hindrance due to ribosome topology. However, our work indicates that there are significant differences, with the exception of region 525, in the dynamic behavior of the rRNA, as judged by changes in K_{ds} , once it becomes part of a normal 30S subunit. The differences may be manifested in either a restriction or enhancement of conformational ranges.

It is very likely that rRNAs go through conformational changes during the various phases of protein synthesis (Kössel *et al.*, 1990; Moazed and Noller, 1986; Leclerc and Brakier-Gingras, 1991). Conformational shifts in some instances may involve ribosomal proteins (rproteins). However, the single stranded conserved regions investigated here are not directly associated with rproteins. Any changes in the

structural and functional behavior of these zones is a reflection of the constraints imposed upon these in terms of their location within the particle and the state of the particle. Our data support the existing evidence that the zones are available for secondary and tertiary intra- or intermolecular interactions with ribosomal or nonribosomal components (Wickstrom, 1983; Moazed and Noller, 1990). Intramolecular interactions may serve as transition "switches". Our DNA/16S RNA complexes in 30S subunits appear to be less than stoichiometric (~0.7:1) (Table 2-3). The binding stoichiometry for all of the DNA probes with free RNA is essentially unit molar. The lower 30S values may be due to transient competing interactions between the probes and other parts of 16S rRNA positioned within the particle. One might view these as interactions that occur in "idling" particles, in which phase transition interactions are stabilized during various stages in protein synthesis.

The binding affinities with DNA probes to the conserved regions investigated here (sequences 520-530, 1396-1404, and 1493-1504) are in the range of those characteristic of initiation factors (Weiel and Hershey, 1981) and antibiotics (Cundliffe, 1980). The K_d for zone 525 in 30S was not significantly different from that of free 16S RNA. Region 1500, on the other hand, showed more than two-fold greater binding affinity when in the 30S conformation relative to that observed in 16S rRNA (Table 2-2). It is of interest to note that sequence within the 1500 zone is required for If3 binding to 30S subunits (Wickstrom, 1983). The implication here is that small antisense DNAs should be effective antibiotics that behave like competitive inhibitors of protein synthesis or ribosome assembly.

Sequences around the 1400 area display a weaker interaction in the 30S particle; there is a two-fold decrease in binding affinities (Table 2-2). Evidently in this zone helix formation is strained because of the constraints imposed by the ribosome on the conformation of the RNA. Electron microscopic studies using avidin binding show that this region is slightly embedded within the 30S particles (Oakes *et al.*, 1986). Baudin *et al.* (1989), using an ethylnitrosourea probe, have demonstrated that the oxygens of the phosphates of bases 1397 and 1399 are less reactive when in the 30S particle conformation than when in 16S RNA. These observations, along with our findings imply, that the 1400 region takes on a different conformation in 30S relative to 16S rRNA. Single stranded RNA zones within the ribosome can clearly take on an array of conformations that can at best be sensed by K_{ds} for hybrid formation. It would be of great value to have a way to characterize these structures fully.

Removal of bases 1401-1404 in 16S rRNA clearly changes the behavior of 30S subunits with regard to DNA probe binding to the area. Altered 30S particles show nearly a two-fold increase in binding affinity relative to normal 30S; however, the binding stoichiometries remain unchanged (Table 2-3, Fig. 2-3). We believe the differences in the sequence of the DNA probes is not a major contributing factor in the apparent binding behavior. For example, even though the same DNA probe is used for binding to either 16S RNA or 30S particles, a significant change in binding characteristics is observed in the case of the 1400 and 1500 zones in normal 30S particles relative to 16S rRNA. The binding

affinity for DNA complementary to the 525 region in normal molecules is different from that observed in altered particles even though the sequence remains unchanged. Furthermore, in spite of DNA probe sequence differences, both the normal and the altered 16S RNA behaved the same with regard to K_{ds} for specific DNA probe interactions with the free RNA. Beyond these considerations, if one uses G+C content to predict the stability of probe interaction, the K_d should be greater for the altered RNA. Furthermore, the calculated ΔG° for the hybridization based upon sequence alone for altered RNA is such that, relative to normal rRNA, the K_d would be expected to be greater than that seen for normal particles (Freier *et al.*, 1986). This is not what we observe. Instead we find that the probe for the altered DNA has a lower apparent dissociation constant. Therefore, base composition alone, when small DNA probes are used, is not a clear predictor of the thermodynamics involved in these interactions. The observed differences could be the result of an unfavorable entropy factor that emerges from fixing the sequence in place. Using the experimentally determined K_{ds} we calculate based on the methods of Breslauer *et al.* (1986), that there is a ΔS reduction of about 25% for binding interactions in altered particles. We take this to mean that the molecule is more rigidly fixed in 30S particles. This may also reduce the number of possible competing interactions with other parts of the RNA.

In vitro translation systems programmed with MS2 message synthesize mainly phage coat protein (129 amino acids; 13,700 Daltons) (Hershey *et al.*, 1981; Sugiyama *et al.*, 1969). When radiolabeled lysine

was used ribosomes missing bases 1401-1404 synthesized approximately 50% less coat protein than normal ribosomes, as determined by counting acid-precipitable radiolabeled proteins as well as polyacrylamide gel electrophoresis. Polyacrylamide/SDS gel analysis of the products reveal that mainly one product, coat protein, is produced by both normal and altered ribosomes. Reduction in translation efficiency by altered ribosomes could result from: a) a reduction in the rate of elongation of protein synthesis or b) premature termination of translation. One could interpret the reduced growth rates in *E. coli* with C1402 to U1402 transitions observed by Jemiolo *et al.* (1985) to support either proposition. More recently this group has provided evidence suggesting that this mutation reduces *in vivo* translational activities by 50% (De Stasio *et al.*, 1988). These authors conclude that such mutations impair the elongation rate of translation. We feel the possibility that the ribosomes are prematurely terminating to be more likely. To discriminate between a rate effect and premature termination, we used [³H] phenylalanine in MS2 mRNA in *in vitro* translations because Phe is coded for predominantly at the beginning of the coat cistron (Figure 2-5). If the defect is one of an altered rate of synthesis, then the Phe incorporation should be equal to that observed when radiolabeled lysine is used. If the defect involves premature termination, then Phe will produce a higher incorporation of label into short peptides assuming initiation is not affected. When one measures just peptide bond formation rates, they can appear near normal because of the location of the [³H]-Phe label. A TCA precipitation assay was used here because it is more sensitive to total

Figure 2-5. Schematic representation of incorporation of [^3H]-Phe (\square) and [^3H]-Lys (\circ) into bacteriophage MS2 coat protein. Part A shows the protein synthesis elongation process. Part B shows the relative position of Phe (\square) in MS2 coat protein as the nascent peptide emerges from the polysome and C shows the placement of Lys (\circ) in emerging MS2 coat protein.

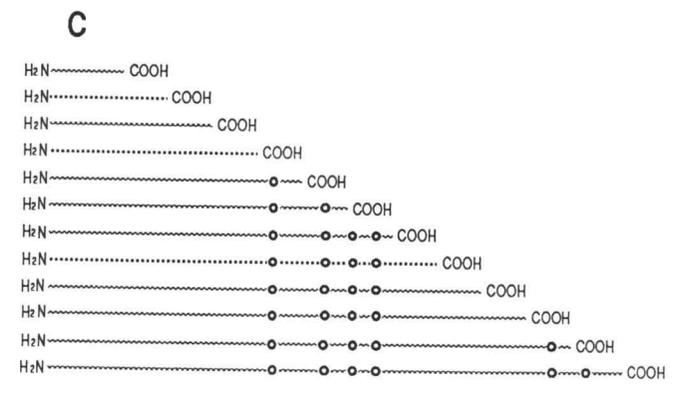
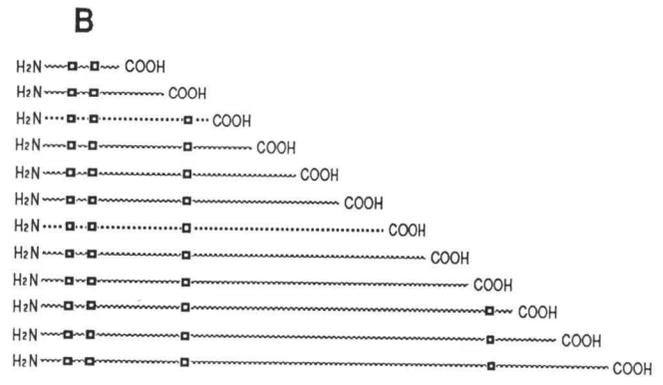
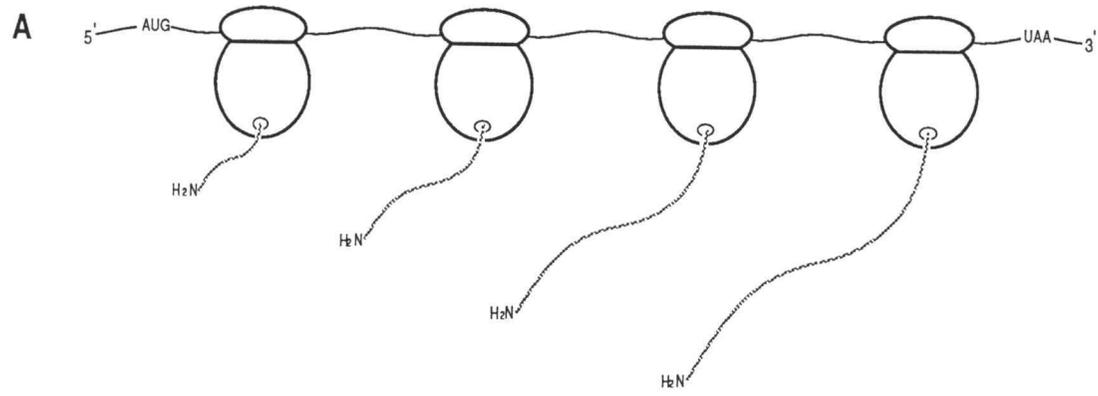


Figure 2-5

peptide bond formation. This is in contrast to polyacrylamide gel electrophoresis, which is most useful for detection of larger complete peptides. The altered ribosomes incorporated labeled Phe with about 85% efficiency relative to normal ribosomes. This is not significantly different from the incorporation observed when using normal 30S subunits and in sharp contrast to what is observed when [^3H]-Lys is employed. This finding eliminates the possibility of a defect in initiation, and strongly supports the interpretation that alteration of the 1400 region causes premature termination. About half of the prematurely terminated polypeptides would not be detected when labeled lysine is used, while the TCA-precipitated small peptides are detectable with the label in the phenylalanine. Since the amount of [^3H]-Phe labeled peptides precipitated using altered 30S is nearly identical to that found for normal ribosomes, we conclude that the rate of peptide bond formation is unchanged.

The anomalous K_d for the 525 DNA in altered 30S raises the possibility of base pairing of the universally conserved sequence ACCGC in the 1400 region to a complement between positions 527 to 531 in the 525 conserved region. Recently others have reported mutations that would disrupt such base pairing; these studies include the five-base deletion or substitution of bases 1400 to 1404 (Hui *et al.*, 1988), the *in vitro* dipeptide formation assay involving a deletion or substitution of G-1401 (Denman *et al.*, 1989), and *in vivo* assay of C-1400 deletion (Thomas *et al.*, 1988). In each case the functional data could be interpreted in terms of premature termination possibly associated with disruption of this putative 525/1400

base pairing. The deletion we have constructed preserves base pairing possibilities of sequence spanning positions 1398-1402 of 16S rRNA; replacing a C with a U produces ACCGU at position 1402. It is generally accepted that GU base pairs occur in RNAs. The separation of the two zones is estimated to be 70Å to 100Å (Moazed and Noller, 1990; Trempe *et al.*, 1982), but they may, at least temporarily, be in contact as part of their known relationship to the A and P sites (Moazed and Noller, 1990; Prince *et al.*, 1982). Furthermore, the streptomycin binding site has been located within the ribosomal decoding site and crosslinked to region spanning bases 1394-1415 (Gravel *et al.*, 1987). The relationship between 530/1400 functional pairing is highlighted by more recent studies showing that resistance to streptomycin can be achieved through alterations at position 523 in the *E. coli* 525 loop (Melancon *et al.*, 1988). These observations lend some support to our finding that alteration in the 1400 region might be expected to affect the binding affinities of DNA probes in the 525 region.

Recently Spirin *et al.* (1987) demonstrated that translocation is accompanied by spatial displacement of some parts of the ribosome and it becomes slightly less compact. Wintermeyer and Gualerzi (1983) reported a slow rearrangement of ribosomal particles during initiation of protein synthesis as a result of association with tRNA and initiation factors (Wintermeyer and Gualerzi, 1983). The hydrogen bonding of the two regions probably sets the structural binding sites for tRNA at the onset of protein elongation. Our proposed 530/1400 interactions could take place in conjunction with the proposed base pairing between 5' and

3'-terminal sequences (Kössel *et al.*, 1990) and conformational switching between 5' end and 915 regions of 16S rRNA (Leclerc and Brakier-Gingras, 1991).

2.6. ACKNOWLEDGMENTS

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CHAPTER 3

IN VITRO* GENERATION AND ANALYSIS OF A SITE-SPECIFIC DELETION IN 16S RIBOSOMAL RNA FROM *ESCHERICHIA COLI

3.1. ABSTRACT

Ribosomal RNAs represent the largest molecular components of ribosomes. They provide a structural framework for the particle and are actively involved in the various phases of protein synthesis. Parts of these molecules are strikingly conserved at the primary structural level. Modified nucleotides are also associated with these highly conserved regions. Here we describe an *in vitro* procedure for manipulating rRNA directly in a way that conserves nucleotide modifications. A four nucleotide deletion was made in a highly conserved zone centered about position 1400 in *Escherichia coli* small subunit 16S rRNA. The nucleotides removed span positions 1401 to 1404. The deletion was made by using synthetic DNAs to target the deletion site for RNase H digestion. Once the targeted nucleotides were removed, the remaining large rRNA fragments were ligated using T4 RNA ligase. Ribosomes assembled with the altered 16S rRNA were able to support peptide bond formation in an *in vitro* translation system primed with a natural mRNA (MS2

RNA). However, a significant decrease in the activity of the particles was observed relative to control.

3.2. INTRODUCTION

Ribosomes are intricate macromolecular complexes composed of two unequal size subunits. In the eubacterium *Escherichia coli*, they have about 50 distinct proteins and three ribosomal RNAs (Wittmann, 1982). The small ribosomal subunit is composed of 21 proteins and a 16S rRNA. The general features of *E. coli* ribosomes are well known. Detailed information regarding the function of various ribosomal protein components is abundant, but clear roles for rRNAs remain to be elucidated. Here we report the development of an approach to identify functional properties associated with the small subunit rRNA.

The primary structure of 16S rRNA from *E. coli* is known and a secondary structure model has been proposed (Woese *et al.*, 1983). Currently ribosomal RNAs are thought not only to provide ribosomal protein binding sites, furnishing a foundation for the assembly of ribosomes, but they also are believed to bear important functional roles in translation (Thompson and Hearst, 1983; Moore, 1988; Dahlberg, 1989). Unfortunately, with the exception of a highly conserved short 3'-terminal sequence centered about position 1536 in eubacterial 16S rRNAs (Shine and Dalgarno, 1975; Steitz and Jakes, 1975), unambiguous functional roles for rRNAs have not been delineated. The 3'-terminal portion of 16S rRNA appears to be important for initiation of protein synthesis and maintenance of translational fidelity (Steitz, 1980; Abdul-Latif and Schaup, 1988). There are other parts of small subunit rRNA which are essentially universal (Pace *et al.*, 1983; Woese *et al.*, 1983). That is, they

have been found in homologous positions in all small subunit rRNAs examined. One such sequence centers about position 1400 in *E. coli* 16S rRNA (Woese *et al.*, 1983). We selected this zone for locus-specific mutagenesis experiments.

The advent of recombinant DNA technology brought with it numerous *in vitro* methods for site-specific mutagenesis (Landick, 1982; Morales and Bagdasarian, 1991). Unfortunately, there are circumstances when these procedures are of limited use. Analysis of 16S rRNA function is an example. This is because rRNA is likely to be multifunctional, being required for both ribosome assembly and subsequent activity in protein synthesis. Difficulty arises when an alteration in the structural gene impacts on a consensus tract required for proper nucleotide modification or nucleolytic post-transcriptional processing. An alteration causing a defect in processing would allow assessment of that functional facet of the molecule, but it would not permit analysis of other putative functions which emerge with proper ribosome biogenesis. The inherent assumption made here is that assembly-required sequences may also be vital for unique steps in protein synthesis. Dual use of transcripts is almost certainly a universal feature of all of the nontranslated RNAs employed in protein synthesis. Comparable tiers of complexity are also likely to occur in RNA viruses which are both economically and biomedically important.

We wished to assess the working aspects of 16S rRNA which are not associated with post-transcriptional alterations of the molecule. Since *in vitro* mutagenesis tends the most opportunistic method for doing this,

a technology to take advantage of fully processed RNAs was required. Here we present an approach designed to permit manipulation of specific segments of mature *E. coli* 16S rRNA. This technique was initially adopted by Y.S.Yoo (1987) and the improvements I have made are discussed here. The procedures are general and applicable to any RNA. Through the use of short synthetic DNAs, RNase H, and T4 RNA ligase, we have been able to construct an altered 16S rRNA with four nucleotides excised between positions 1400 and 1405. This altered 16S rRNA is competent in *in vitro* reconstitution systems (Traub and Nomura, 1969). The small subunit particles produced support protein synthesis in an *in vitro* translation reaction primed with MS2 mRNA. However, altered ribosomal particles show a significant decrease in protein synthesis capability.

3.3. MATERIALS AND METHODS

3.3.1. General Preparations

Mid-log phase *E. coli* (D-10) cells were the source of ribosomal components. The cells were disrupted with a French pressure cell at 15,000 psi. Ribosomal RNAs were isolated from phenol-extracted cell lysates by zone ultracentrifugation in 15-30% sucrose gradients made in TSM buffer, containing 10 mM Tris-HCl (pH 8.0), 3 mM succinic acid, 10 mM MgCl₂, and 3mM β-mercaptoethanol (Schaup *et al.*, 1970). Ribosomal 30S subunits were isolated by zone ultracentrifugation and 30S proteins were extracted with acetic acid as described by Hardy *et al.* (1969). Protein concentration was determined using the Bradford procedure (Bradford, 1976). Cytidine 3',5'-bisphosphate 5'-[³²P]pCp was purchased from NEN or synthesized as previously described (England *et al.*, 1980) by using 3'-CMP and γ[³²P]ATP (7000 mCi/mMol, ICN Radiochemicals). Ribonuclease H from *E. coli* was a generous gift from R.J. Crouch. The following products were obtained commercially: calf intestinal alkaline phosphatase (E.C. 3.1.3.1) (NEN), T4 polynucleotide kinase (E.C. 1.7.1.78) and T4 RNA ligase (E.C. 6.5.1.3) (BRL), RNase T1 (E.C. 3.1.4.8) (Sankyo), RNA sequencing system (BRL), *E. coli* tRNA (Calbiochem), ultrapure acrylamide and N,N'-methylene bisacrylamide (BRL), electrophoresis grade urea (Schwarz/Mann), Sephadex G-50 (Pharmacia), and Matrex gel PBA-30 (Amicon Corporation).

3.3.2 Synthesis of DNA Oligomers

DNA oligomers were synthesized on an Applied BioSystems DNA Synthesizer Model 380A using activated nucleoside phosphoramidites (Caruthers, 1985; Matteucci and Caruthers, 1981). The completed DNA oligomers were purified by reverse-phase ion pairing high pressure liquid chromatography (Haupt and Pingoud, 1983), or by extraction from polyacrylamide gels as described earlier with some minor modifications (Sanchez-Pescador and Urdea, 1984).

3.3.3. 5'-and 3'-End Labeling

The 5'-terminal phosphates of RNAs were removed by using calf intestinal alkaline phosphatase. RNA was incubated for 30 minutes at 37°C with 1 unit of enzyme per 2.77 A₂₆₀ units of RNA in phosphatase buffer containing 100 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, and 0.1 mM ZnCl₂. When required, the dephosphorylated RNA and DNA oligomers were 5'-end-labeled with γ [³²P]ATP and T4 polynucleotide kinase according to Sambrook *et al.* (Sambrook *et al.*, 1989). RNA was 3'-end-labeled using 5'-[³²P]pCp and T4 RNA ligase (Meyhack *et al.*, 1978). The labeled RNA was mixed with two volumes of tracking dye (Peattie, 1979) and was purified by polyacrylamide gel electrophoresis. Excised gel fragments were eluted by the method of Cory *et al.* (1972). Samples to be removed from gels were located either by autoradiography, UV

shadowing, or with the aid of an AMBIS beta scanner (Automated Microbiology Systems, Inc., San Diego, CA).

3.3.4. Deletion Construction

Dephosphorylated 16S ribosomal RNA was cleaved at the 1400 region by RNase H upon hybridization with site specific complementary oligodeoxyribonucleotides. Two sets of DNA/RNA hybrid experiments are necessary to obtain the 4-nucleotide deletion. One of the DNA oligomers was complementary to a sequence in 16S rRNA shifted a few bases downstream of the other DNA in 16S rRNA. Hybridizations were performed under conditions similar to those described by Donis-Keller (1979). Preparative hybridization reactions (40-50 μ l) contained approximately 100 μ g 16S rRNA, 10 μ g DNA oligomer and an excess of *E. coli* tRNA (5-10 x mass excess to 16S rRNA) in 50 mM Tris-HCl (pH 8.3), 25 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, and 30 μ g/ml bovine serum albumin. The tRNA was added to diminish nonspecific loss of rRNA in subsequent processing procedures. The reaction mixture was preincubated, at 50°C for 20 minutes and then for 30 min at 37°C to allow annealing of the DNA oligomer. *E. coli* RNase H (0.7 units/ μ g RNA substrate) was added and the digestion allowed to proceed for 50 minutes at 37°C. RNA fragments were separated on an acrylamide/urea gel and electroeluted from the gel by using an Elutrap apparatus (Schleicher & Schuel, Keene, NH.) under conditions described by Zassenhaus *et al.*

(1982). This elution method was necessary to eliminate any residual RNase H and polyacrylamide.

The steps required for generating deletions within the universally conserved region positioned about residue 1400 in *E. coli* 16S rRNA are summarized in Figure 3-1. Each stage in the process was monitored by denaturing polyacrylamide gel electrophoresis of the products of each reaction. Dephosphorylation of the 5'-terminus of 16S rRNA was required to prevent circularization of 16S rRNA during subsequent ligation reactions. The synthetic DNA probes hybridized to the phosphatased 16S RNA were nine oligonucleotides long and complementary to nucleotides spanning 1396 to 1404 and 1399 to 1407. The DNAs are identified as probes 1396 and 1399, respectively (Table 3-1). A computer search of 16S rRNA revealed partial repeats of four or more continuous bases beginning at positions 51_D, 174_D, 269_D, 313_D, 327, 400_D, 501_D, 718, 734_D, 808_D, 878_D, 931_D, 970_D, 1207_D, 1227_D, 1394, 1408_D, 1533. The longest repeat was six bases in length and the positions marked with a subscript D are located in proposed double-stranded regions of the molecule (Woese *et al.*, 1983). Complementary zones in double stranded regions cannot hybridize with our DNA probes under the hybridization conditions used here. Undesirable cleavages in single stranded areas of homology were eliminated by increasing the stringency of the hybridization by performing RNase H digestions at 37°C. *E. coli* RNase H concentration was adjusted (0.7 units/μg of RNA) to limit RNA cleavage to one phosphodiester bond within the target site.

Figure 3-1. Deletion/ligation strategy paradigm for construction of altered 16S rRNA. Diagram shows the use of two DNA probes for RNase H cleavages and a looped DNA joining probe. Upright arrows under the RNA indicate the RNase H cleavage sites. "Lg" refers to long fragments and "Sh" refers to short fragments. The * indicates the location of [³²P]. The small triangle below the completely reconstructed RNA (V) shows the deletion site. Roman numerals indicate separate steps in the process.

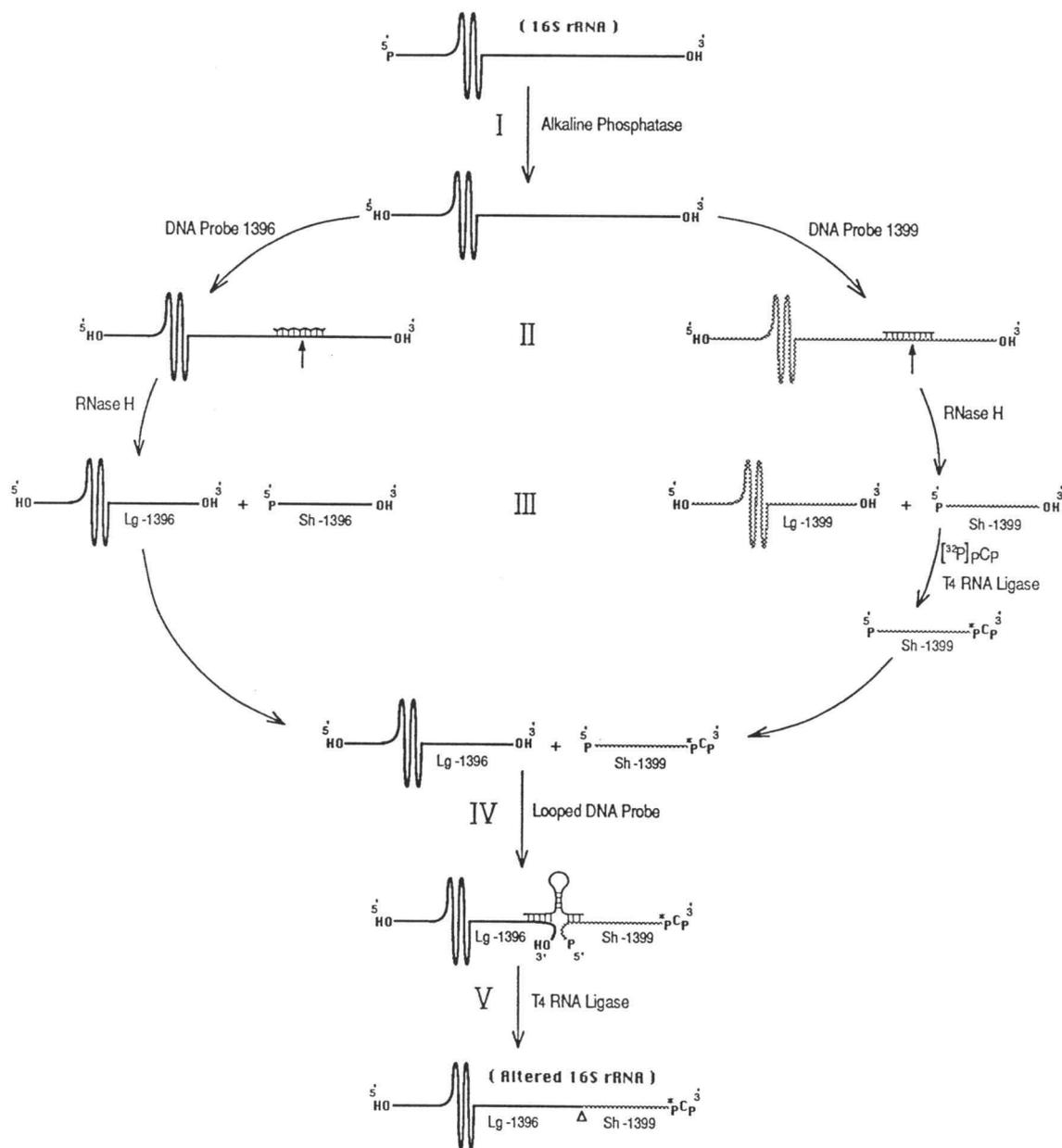


Figure 3-1

TABLE 3-1DNA Probes Alignment to 1400 Region of 16S rRNA^{a,b}

DNA 1396	T-G-T-G-G-C-G-G-G
DNA 1399	G-G-C-G-G-G-C-A-G
16S rRNA	G-U-A-C-A-C-A-C-C- <u>G-C-C-C</u> -G-U-C-A

^aDNA probe sequences shown in 3'-5' direction.^bSequence of 16S is shown 5' to 3' beginning at position 1392.
Underline indicates nucleotides targeted for removal.

Selected 3'-end labeled small RNA fragments were ligated to a dephosphorylated 5' large fragment by using T4-RNA ligase. The two RNA molecules (30 pmole of 5'-large fragment, 8 pmole of 3'-small fragment) were first jointly hybridized to a 17-nucleotide DNA in 0.1 ml reaction volume for 20 minutes at 50°C and then at 37°C for 1 hr. The ligation buffer consisted of 50 mM Hepes (pH 7.5), 20 mM MgCl₂, 3.3 mM dithiothreitol, 10% (v/v) dimethylsulfoxide, 15 µg/ml BSA and 15 µM ATP. T4 RNA ligase (80 µg/ml) was added to the mixture and the incubation temperature was kept at 10°C for about 12 hrs. Phenylboronate agarose (PBA-30) column chromatography (Pace and Pace, 1980) and subsequent ultracentrifugation were employed to separate the religated 16S rRNA from nonligated RNA fragments. PBA columns were run at room temperature, and RNA that did not bind was eluted with application buffer: 50 mM Hepes, pH 8.5/10 mM MgCl₂/0.2 M NaCl at a flow rate of 0.5 ml/min. Bound large fragment RNAs were eluted by using 0.1 M sorbitol in the application buffer. Fractions corresponding to sample peaks were ethanol precipitated onto a CF11 column (Cellulose Powder, Whatman). Columns holding ligated 16S rRNA were washed in 80% EtOH. The RNA was eluted in H₂O and separated from non-ligated 3'-small RNA fragments by sucrose gradient ultracentrifugation (190,000 xg for 90 minutes at 4°C in a Sorvall TV865 rotor). Control 16S rRNA was taken through the entire digestion/ligation procedure in the absence of DNA.

3.3.5. RNA Sequence Analysis

Nucleic acids to be sequenced were either 3'- or 5'-end labeled and analyzed by the partial enzymatic cleavage methods (Donis-Keller *et al.*, 1977) using site-specific ribonucleases purchased from BRL in the form of a sequencing kit.

3.3.6. Reconstitution of 30S Ribosomes

Conditions for *in vitro* total reconstitution included preincubation of 16S rRNA for 30 minutes at 42°C in 10 mM Tris-HCl (pH 7.6), 20 mM MgCl₂, 0.3 M KCl, and 6 mM β-mercaptoethanol (Traub and Nomura, 1969; Amils *et al.*, 1978). Subsequent to the addition of ribosomal proteins, incubation was continued for another 30 min. Formation of assembled 30S particles was confirmed by sucrose gradient ultracentrifugation. To ascertain the presence of all small subunit ribosomal proteins, rRNA was reconstituted into ribosomes with either [³H] or [¹⁴C] lysine labeled rproteins. We made three reconstituted 30S mixtures to compare experimental ribosomes with control ribosomes. These mixtures were: control reconstitution with [¹⁴C] protein (C_c), control reconstitution with [³H] protein (C_h), and experimental reconstitution with [³H] protein (E_h). The C_c reaction was split into two fractions. Following reconstitution, one C_c fraction was mixed with a C_h and one with E_h reaction for C_h/C_c and E_h/C_c ratio determination. Total 30S rprotein composition of the mixtures was analyzed electrophoretically in polyacrylamide gels

(Hardy *et al.*, 1969) by radioassay of gel slices using the procedures described by Cowgill De Narvaez and Schaup (1979). The ratio of ^{14}C to ^3H for each fraction was determined and normalized by applying the relative mass equation [$N_{\text{RI}} = ({}^3\text{H}/{}^{14}\text{C})({}^{14}\text{C}_{\text{total}}/{}^3\text{H}_{\text{total}})$]. These ratios give a reliable comparison between the two samples. If there is no difference between the samples, the N_{RI} value should be one; a number less than one shows a reduction in incorporation of the labeled protein in the experimental reaction. A number greater than one would indicate increased incorporation of the protein relative to controls.

3.3.7. *In vitro* Protein Synthesis

In vitro translations were done in the presence of purified factors required for protein synthesis (initiation, elongation, and termination), MS2 mRNA, and ribosomes by using previously described methods (Van Dieijen *et al.*, 1975; Hershey *et al.*, 1981). In reaction mixtures with a total volume of 0.1 ml and containing 1 mM ATP, 0.12 mM GTP, 5 mM phosphoenolpyruvate, 0.1 μg pyruvate kinase, and 17 μg tRNA in 40 mM Tris-Acetate (pH 7.6)/5 mM magnesium acetate, 50 mM KCl, 12 mM NH_4Cl /6 mM β -mercaptoethanol, the incubation was for 30 minutes at 37°C. The level of incorporation of either [^3H]-Lys or [^{14}C]-Lys (New England Nuclear, Wilmington, DE) into trichloroacetic acid-insoluble material was measured according to Mans and Novelli (1961).

3.4. RESULTS

3.4.1. RNase H Specificity

To ascertain the specificity of RNase H cleavages, 16S rRNA digestion in the presence of specific complementary DNA was done under conditions where DNA concentration was limiting. In this situation a complete array of digestion products could be observed if cleavages were occurring at positions other than the target site. In the presence of optimal amounts of DNA, secondary cleavages could be masked, leaving only the dominant expected cleavage product. This is a hybridization stringency control as well as a control for nonspecific nuclease contamination in our enzyme preparations. Results of such experiments are shown in Figure 3-2. Two bands, as expected, were observed in the 16S rRNA zone of the gel. The upper band is uncleaved 16S rRNA and the smaller product in the lower portion of the polyacrylamide gel is of the expected size, relative to 16S rRNA, of the high molecular weight fragment. Cleavages were observed only in the presence of both DNA and RNase H. The relative band intensities between the high and low molecular weight products within the gel are a measure of the digestion efficiency. Under optimal conditions the cleavages were essentially 100% efficient.

The products of the digestions in the presence of the individual DNA probes were purified by polyacrylamide gel electrophoresis as described in Materials and Methods. The termini of cleavage products

Figure 3-2. RNase H digestion products of 16S rRNA in the presence of DNA probe 1396. RNA bands were visualized by staining with ethidium bromide. Lane 1: 16S rRNA marker. Lane 2: High molecular weight 16S rRNA RNase H cleavage product. Lane 3: 16S rRNA incubated with RNase H in the absence of DNA. Lane 4: 16S rRNA incubation with DNA in the absence of RNase H. Migration towards the anode is from top to bottom. The polyacrylamide gel (10cm x 10cm x 0.8mm) was a 2.8% and run at 4mA for 2 hr at 10°C.

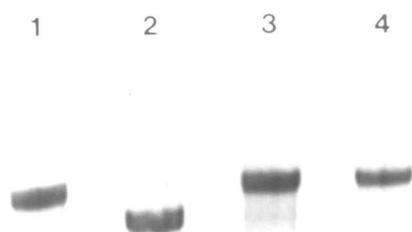


Figure 3-2

were sequenced as described in Materials and Methods prior to use. Sequence analysis of the 3' end of the large fragment produced under our conditions showed that the cleavage site in the presence of probe 1396 was between C1400 and G1401 (Yoo, 1987). The fragments generated by using DNA 1399 had a cleavage between positions C1404 and G1405 (Yoo, 1987).

3.4.2. Ligation of rRNA Fragments

T4 RNA ligase mediates an ATP-dependent ligation reaction (Meyhack *et al.*, 1978). The enzyme requires a free 3'-hydroxyl group which functions as an acceptor and a 5'-phosphate group that serves as a donor (Uhlenbeck and Gumpert, 1982). The pCp is used to label the RNA, and its 3'-phosphate also serves as a 3'-end blocking group on the small RNase H digestion product. Prior treatment of the 16S rRNA with alkaline phosphatase leaves the larger fragment's 5'-end without a phosphate, making it an unsuitable donor in T4 RNA ligase reactions. Because of the presence of a 3'-terminal phosphate and the absence of a 5' terminal phosphate, T4 RNA ligase cannot catalyze ligation of two large or small fragments; likewise the smaller fragment cannot become attached to the 5' end of the large fragment. RNase H cleavage leaves the large fragment with 3'-hydroxyl and the small fragment with 5'-phosphate. These are the only appropriate substrates present in the ligation reaction. This means that there is only one possible ligation product and it will have the same polarity as the original 16S rRNA. The

appropriate choice of large and small RNase H digestion products permits construction of a 4 base deletion in an otherwise fully mature and complete 16S rRNA. To enhance ligation efficiency, a synthetic DNA (5'-TGAGAACAAGTTATGTG-3') was designed to hybridize to the selected large and small ligates in positions that bring and hold the fragments ends close together to optimize the ligation (Fig. 3-1 step IV). The 3' fragments have one base that is not hybridized and the 5' fragment has two free bases. To monitor the ligation, the small fragment (138 nucleotides) was 3'-end labeled with [³²P]pCp. Following incubation with T4 RNA ligase and the larger fragment (1400 nucleotides), the mixtures were analyzed electrophoretically on denaturing polyacrylamide gels. The appearance, on autoradiographs of the gels, of [³²P] labeled product in the 16S rRNA (1542 nucleotides) zone on a gel provides a quantitative measure of ligation efficiency. In the presence of the linking DNA there was a five-fold increase in ligation efficiency relative to ligation without it (Fig. 3-3, reactions 1 and 3). Lowering the divalent ion concentration reduces ligation efficiency somewhat (Fig. 3-3, reaction 2).

An additional strategy was applied to conserve material and purify ligated RNAs. Following ligation the reaction mixture was passed through a phenyl boronate agarose column (PBA-30). Phenyl boronate agarose chromatography separates polyribonucleotides on the basis of the presence of 2',3'-cis-diol termini (Pace and Pace, 1980). Under specific conditions, these columns can be used for separations involving molecules of the size of 16S rRNA (Pace and Pace, 1980). The pCp

Figure 3-3. Deletion construction. Autoradiograph insert is of a polyacrylamide gel showing the ligation of 3'-[³²P]pCp-labeled short fragment to unlabeled large fragment (Figure 3-1 step V). The bar graph shows ligation efficiencies as a percent of total ³²P that appears in the 16S zone on the polyacrylamide gel. M is a 5'-end labeled 16S rRNA marker. Reaction 1: ligation of the fragments in the absence of the looped DNA probe (Figure 3-1 step IV). Reactions 2 and 3 are ligated products in the presence of the looped DNA probe in with 10 mM MgCl₂ and 20 mM MgCl₂, respectively, in the buffer.

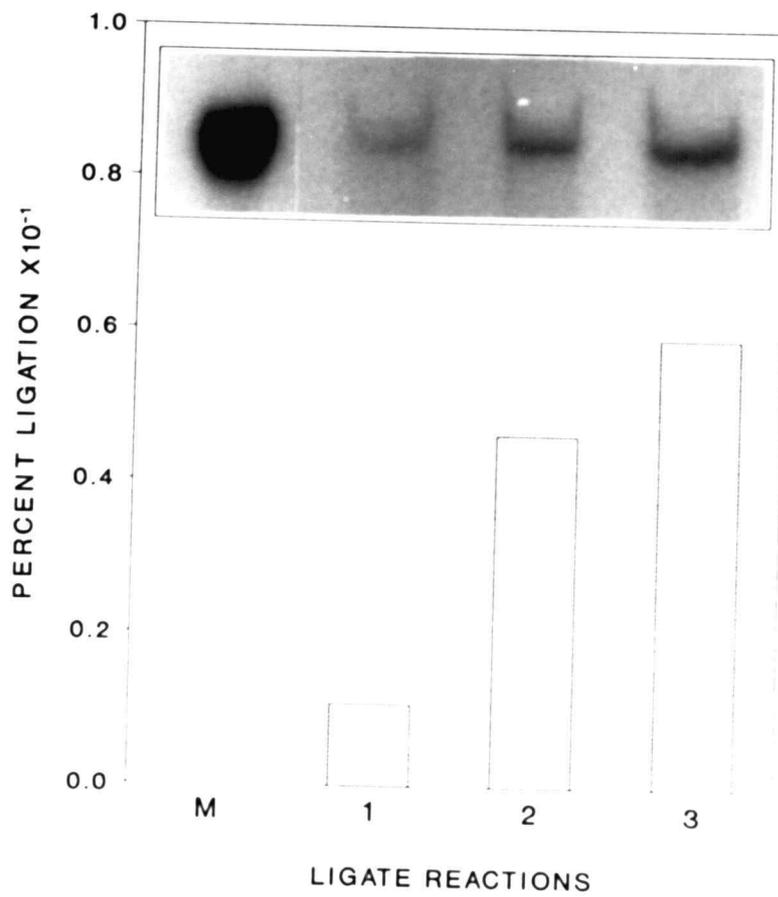


Figure 3-3

blocking group at the 3'-end prevents 16S rRNA constructs from binding to the column, whereas unreacted large fragment with its free cis-diols binds to the column. Unreacted pCp 3'-end labeled small fragment also does not bind to the column because of the presence of the 3'-phosphate. This fragment is very small relative to the 16S ligate and is easily removed by zone ultracentrifugation before the 16S rRNA constructs are used in ribosome reconstitution experiments (Fig. 3-4). Unused large and small fragment were recovered and recycled after concentration and dialysis into ligation buffers.

3.4.3. RNA Construct Sequence Conformation

The reconstructed RNA molecule should have four nucleotides removed. To confirm the sequence of this deletion quickly and easily without using appreciable amounts of the product, a nine-base DNA oligomer complementary to the new region in the construct was synthesized. The new DNA nanomer (5'-TGACGGTGT-3') and the DNA probe 1396 (5'-GGGCGGTGT-3') were 5'-end-labeled using polynucleotide kinase and γ [³²P]ATP. The [³²P]-labeled DNA oligomers were respectively hybridized to the reconstructed RNA molecule and then subjected to nuclease S1 attack (Rahman and Schaup, 1990). The hybridization conditions were as described for RNase H digestions. Nuclease S1 will not attack double-stranded nucleic acids under these conditions (Berk, 1981). Therefore, if the anticipated ligations had occurred, one would expect probe 1396 (5'-GGGCGGTGT-3') to be

Figure 3-4. Recovery and recycling of ligates and RNA fragments. Panel A shows elution profile of ligated fragments and residual small fragment from a PBA-30 column. Arrow indicates the sorbitol buffer elution front for bound large RNA fragment. Panel B shows the separation of ligated molecules from 3'-end labeled short fragments by zone ultracentrifugation in a 15-30% sucrose gradient. Samples from the PBA-30 column were ethanol precipitated and were resolubilized in 10mM Tris-HCl, pH8.0/0.25 mM EDTA/1M urea, and centrifugation time was 1.5 hr at 190,000 x g in a Sorvall TV865 rotor.

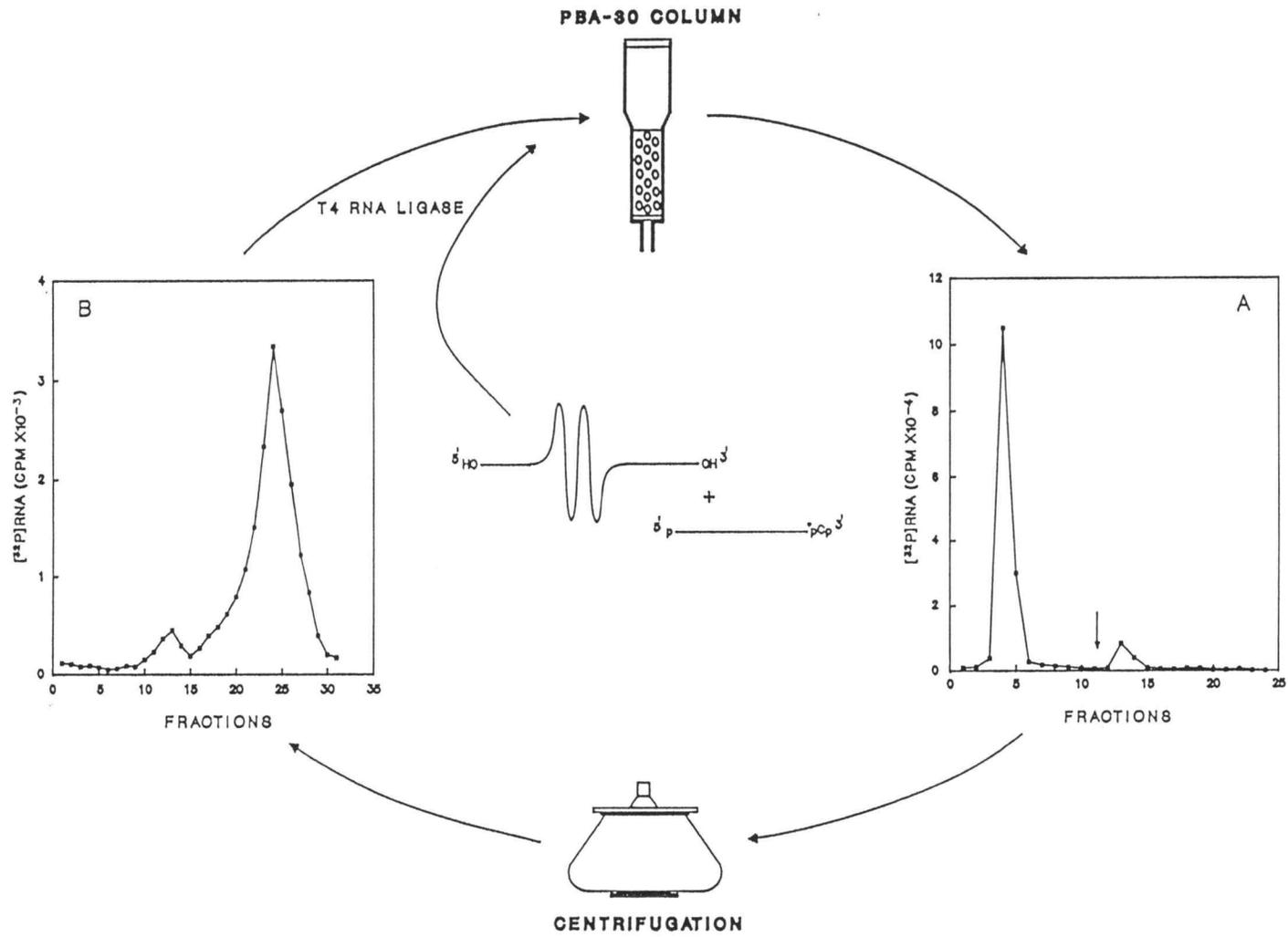


Figure 3-4

destroyed because its 5'-end will not have a complement with which to hybridize. The nanomer (5'-TGACGGTGT-3') should be fully complementary with the altered RNA and thus protected. Analysis of these digestion reactions on 20% polyacrylamide gels (Figure 3-5, lane 1) support the conclusion that the recovered product was the desired RNA construct. As anticipated, DNA probe 1396 complementary to normal 16S rRNA was not protected when hybridized to the new 16S rRNA (Figure 3-5, lane 2). We use this procedure routinely to confirm the quality of constructs missing bases 1401-1404.

3.4.4. *In vitro* Reconstitution and Protein Synthesis

Small 30S ribosomal subunits assembled with [³²P] labeled 16S rRNA deletion constructs are indistinguishable from 30S reconstituted with normal 16S rRNA when analyzed by zone ultracentrifugation in sucrose gradients (Figure 3-6). Further analysis of the reconstituted particles using a protein double-labeling technique (Cowgill De Narvaez and Schaup, 1979) shows that all of the rproteins are present in the altered 30S. Here experimental 16S rRNA constructs were reconstituted into 30S particles with [³H]-Lys labeled ribosomal proteins. Normal 16S rRNA was used in 30S reconstitutions with [¹⁴C]-Lys labeled ribosomal proteins. Following reconstitution the control and experimental reconstituted particles were mixed and the protein was extracted with acetic acid. The protein mixtures were separated by polyacrylamide gel electrophoresis as described by Hardy *et al.* (1969). Following

Figure 3-5. Autoradiograph showing S1 nuclease mapping to confirm deletion in reconstructed molecules. DNA probes were 5' [³²P]-labeled as described in Materials and Methods. Lane 1: DNA probe (5'-TGAC[^]GGTGT-3'); [^] indicates the position of 4-nucleotide deletion; hybridized to altered 16S rRNA and then subjected to S1 nuclease digestion. Lane 2: DNA probe 1396 (5'-GGGCGGTGT-3'), complementary to normal 16S rRNA, hybridized to altered 16S and subjected to S1 nuclease digestion. Lane 3: DNA (5'-TGAC[^]GGTGT-3') subjected to S1 nuclease digestion in the absence of any rRNA. Lane 4: DNA (5'-TGAC[^]GGTGT-3') marker. The digestion and hybridization conditions were as described in Materials and Methods. The specific activity for each of the DNAs was adjusted to 1×10^7 cpm/ μ g by the addition of unlabeled DNA; 0.02 μ g of RNA were present in each reaction mixture.

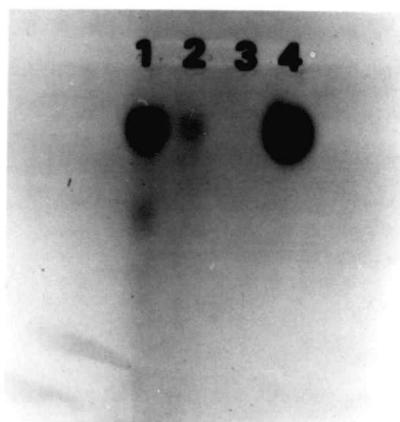


Figure 3-5

Figure 3-6. Sedimentation profile of particles reconstituted with altered 16S rRNA. Unfilled boxes (-□-) show the distribution of 3'-end [³²P]pCp-labeled altered 16S rRNA; the filled boxes (-■-) show the profile of reconstituted 30S particles using non gel purified normal 16S rRNA that serves as an internal standard. The arrow indicates the location of 16S rRNA. Sedimentation gradients were fractionated by piercing the bottom of the centrifuge tube and collecting ~1.4 ml fractions. The separation was achieved by using a Sorvall TV850 rotor. The run time was 2 hours at 45,000 rpm in a 15% to 30% sucrose gradient made in TSM buffer.

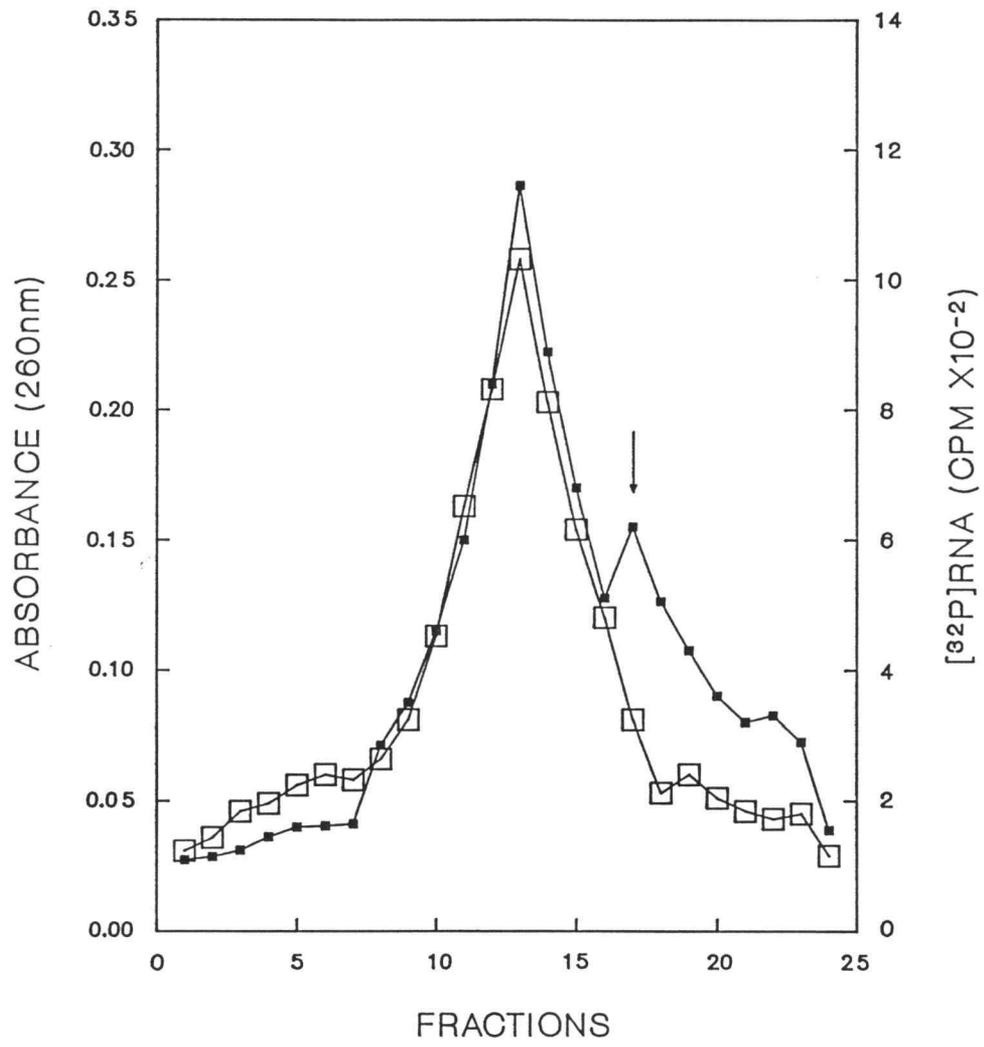


Figure 3-6

electrophoresis the gel was sliced and slices were analyzed for ^{14}C and ^3H content. The isotope ratios were used to calculate the relative mass fraction of protein in each slice. If the two different protein samples are identical the mass fraction number will be 1. Here the calculated mass fractions did not vary significantly from one indicating that a normal complement of protein is present in the 30S assembled with experimental 16S rRNA (Figure 3-7). Calculated mass fractions would deviate significantly from one in zones analyzed in the polyacrylamide gel if a protein or proteins were missing in the particles reconstituted with altered 16S.

The competency of altered 30S particles was also analyzed in an *in vitro* translation system programmed with MS2 mRNA. Peptide bond formation was assayed by TCA precipitation of protein which was collected on GF/C glass filters. Small subunit particles reconstituted with altered 16S rRNA were compared for activity in translation systems with particles containing normal RNA as described in Materials and Methods. The control 16S rRNA was taken through the entire digestion/ligation process but without DNA. The level of incorporation of labeled lysine into polypeptides using altered ribosomes represented approximately 50% of that which resulted from normal ribosomes. The experimental to control (E/C) ratio was 0.51 ($\pm 5\%$) representing a mean of two replicates and was calculated by using the appropriate individual control values. A more complete analysis of this 16S rRNA construct can be found in the accompanying report.

Figure 3-7. 30S proteins mass fraction ratios from polyacrylamide gel separation of proteins. a) (-■-) ^3H -labeled control 30S rproteins/ ^{14}C -labeled control. b) (-□-) ^3H -labeled 30S rproteins reconstituted with the altered 16S rRNA/ ^{14}C -labeled control. Dotted lines indicate \pm one standard deviation.

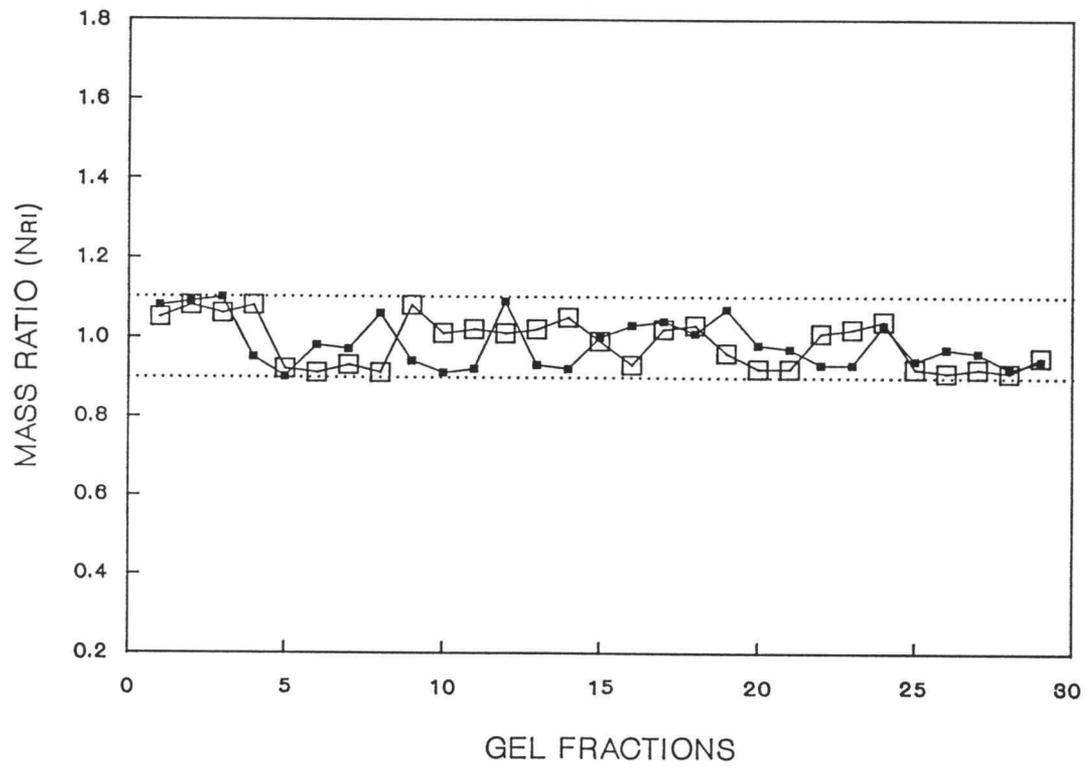


Figure 3-7

3.5. DISCUSSION

Bacteriophage T4 RNA ligase has been widely used for 3'-terminal labeling of RNA using nucleoside 3',5'-bisphosphate as a donor molecule (England and Uhlenbeck, 1978; Keith, 1983; England *et al.*, 1980). The enzyme is also useful for joining short oligonucleotides (Krug *et al.*, 1982; Middleton *et al.*, 1985) up to the size of transfer RNAs (Wang *et al.*, 1981; Uhlenbeck, 1983; Schulman and Pelka, 1985). However, a comprehensive investigation of factors required for efficient ligation of large RNA molecules has not been reported. Improving the efficiency of the ligations that complete the 16S rRNA reconstruction described in this work were the most challenging facet of the procedure. When working with small RNA molecules, the terminal nucleotide as well as secondary structure have an impact on the yield of joined product (McLaughlin *et al.*, 1985, England and Uhlenbeck, 1978). Because of this, sequence can become a limitation when manipulation of a specific zone within a large RNA molecule such as the 16S rRNA is necessary. The secondary structure of the molecules to be ligated must also be considered. RNA ligase works best when both donor and acceptor ends are single stranded having at least one nucleotide extend beyond a double stranded conformation (Bruce and Uhlenbeck, 1978). Blunt ligations do not work well (Meyhack *et al.*, 1978; Cranston *et al.*, 1974). As others have reported, we also found that RNA ligase favors small oligonucleotides as substrates. Very small amounts of oligonucleotide contaminant in preparations become the dominant substrates in RNA ligase reactions.

For instance, when analyzed by polyacrylamide gel electrophoresis, 16S rRNA isolated from sucrose gradients shows very low specific activity when labeling is done with [^{32}P]pCp. Most of the radioactivity is incorporated into low molecular weight contaminating fragments, even though staining of the gel shows only traces of small oligonucleotides in the preparations. When 16S rRNA is purified from denaturing polyacrylamide gels, it becomes an excellent substrate for the labeling reaction because the competing small oligonucleotides have been removed. Therefore, along with sequence and secondary structural considerations, it is extremely important to prepare samples of high purity when attempting constructions of the type described here.

Here we have introduced some techniques for improving the yield of large RNA constructs. The looped (Figure 3-1, step IV) probe method was intended to bring the ends to be ligated into close proximity and mimic an optimal secondary structure for ligation within the constraint of the single stranded zone we worked with. A five fold increase in ligation efficiency was observed by using this strategy. Greater efficiency may be possible with different sequence environments. Accordingly, cycling and recycling substrates through phenyl boronate agarose columns in conjunction with zonal ultracentrifugation minimized the need for continuous preparation of large quantities of substrates. This is a particularly useful time- and cost-saving technique given that preparations of RNA constructs requires substrates of high purity. Although these methods were applied here to produce specific 16S rRNA

constructs, we believe that they are general and may be used for manipulation of any large RNA.

The direct RNA manipulation approach taken in this study has important advantages over conventional DNA mutagenesis techniques. We preserve all of the post-transcriptional modifications outside of the target zone. Site directed DNA mutagenesis of 16S rRNAs that are transcribed from DNA *in vitro* produces RNAs that contain additional sequence differences outside of the target zone (Denman *et al.*, 1989). These RNAs also lack all the modified bases that are found in normal 16S rRNA (Noller, 1984). Recent isolation of methyltransferases specific for bases G966 and C967 in 16S rRNA (Négre *et al.*, 1989) gives some hope of completing modifications *in vitro*. *In vitro* functional studies are attractive because they have the advantage of excluding normal *in vivo* ribosome background. This latter point is an important consideration in *in vivo* mutagenesis experiments (Zwieb *et al.*, 1986; De Stasio *et al.*, 1989).

When the 16S rRNA with four bases ($G_m^4 C_m CC$, 1401 to 1404) deleted was used in *in vitro* reconstitution experiments, 30S particles were recovered. The proteins normally associated with the particles were present on these altered particles. This does not mean that orientation of the proteins relative to one another has not changed in some subtle way. It is, however, worth noting that the zone manipulated in this work is not known to directly associate with ribosomal proteins (Lake, 1985; Oakes *et al.*, 1986; Brimacombe and Stiege, 1985). Removal of part of this highly conserved region clearly affects the functional characteristics of

ribosomes. Acid precipitable counts recovered in *in vitro* translation show a 50% decrease in apparent translation efficiency. The impairment of the altered ribosomes is a reflection of the critical role the 1400 region plays in protein synthesis processes. The disposition of the 1400 zone on the surface of the 30S particle (Oakes *et al.*, 1990) and its protection from chemical attacks by tRNA binding (Moazed and Noller, 1990) suggest that this region is in the proximity of tRNA binding domains. Impairment of the altered ribosomes in protein synthesis could be due to an inability to form and/or maintain a proper peptidyl site (Prince *et al.*, 1982; Moazed and Noller, 1990). Removal of four nucleotides at this region could alter the peptidyl site in a way that destabilizes retention of nascent protein on the ribosome once it begins to emerge from the particles during synthesis.

3.6. ACKNOWLEDGMENTS

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APPENDIX

APPENDIX

Calculation of standard errors for the apparent dissociation constants and the binding stoichiometry:

The apparent dissociation constant for DNA probe binding to 16S rRNA is calculated from the slope line of the Scatchard plot where

$$K_d = -\frac{1}{\text{slope}} = \frac{n}{nK_a},$$

n = ordinate intercept and K_a = the binding association constant.

The binding stoichiometry is determined from the ordinate intercept.

The standard error of the simple linear regression is estimated from the equation

$$SE = \frac{s}{\sqrt{\Sigma(x-\bar{x})^2}}, \text{ s is the standard deviation } = \sqrt{\frac{\Sigma(y-\hat{y})^2}{n-2}}, \text{ n =}$$

the number of experimental data and ($\hat{y} = a + bx$) is the equation of the least squares line.

Figure A-1. Relative mass ratios of *in vitro* translation products analyzed on SDS-polyacrylamide gel (see Figure 2-4). N_{RI} indicates the ratio of [3H] lysine counts of the products, from using altered ribosomes (-■-) or normal ribosomes (-□-), to [^{14}C] lysine incorporation. The [^{14}C] lysine is used with the normal ribosomes. N_{RI} for each fraction is calculated by deviding 3H into ^{14}C and multiplying into the inverse of the same ratio for the total counts recovered from all the fractions collected. The arrow indicates the position of MS2 coat protein. The dotted lines mark one standard deviation. Protein standards (Bio-Rad) were run in the adjacent lane and visualized by protein-specific stains.

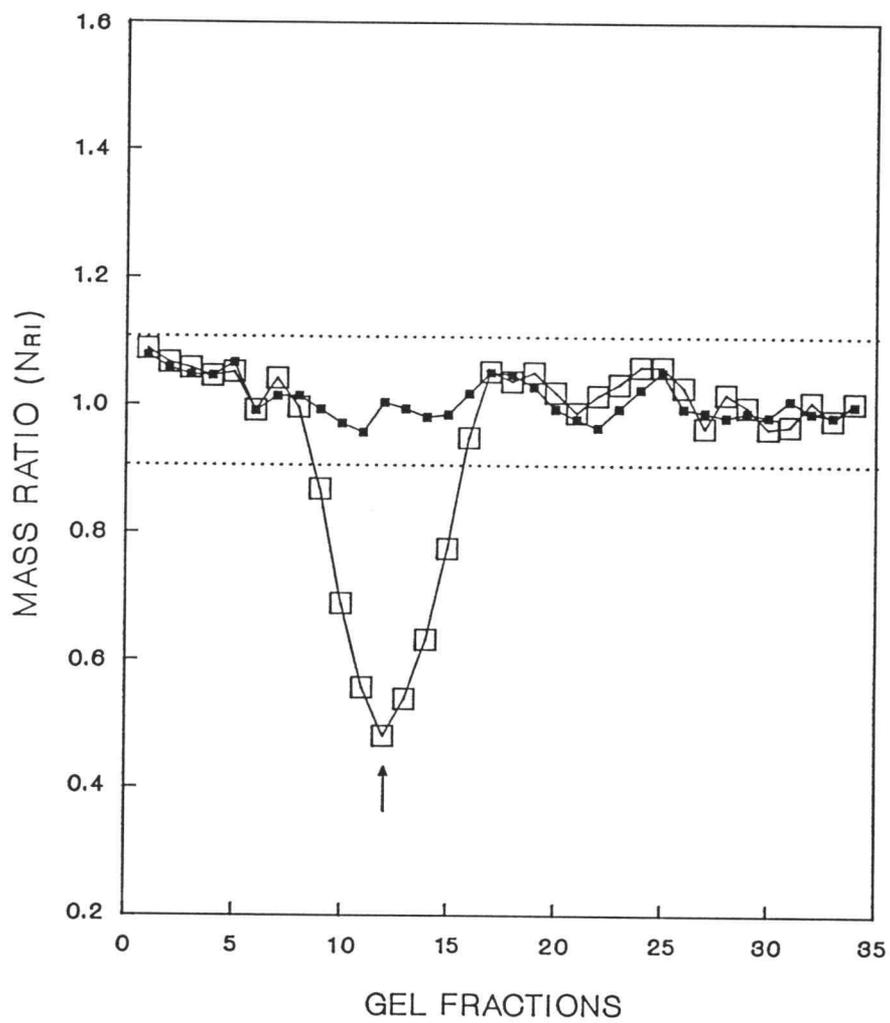


Figure A-1

Figure A-2. Autoradiograph of enzymatic digestion pattern of 16S rRNA and the two intact 3' small fragments generated by using DNA probe 1396 (lane 1) and probe 1399 (lane 2). RNA was 3'-end labeled [³²P]pCp. Lane G is generated by partial digestion of 16S rRNA with RNase T1. Lane U+A is the product of digestion of 16S rRNA with RNase Phy M. Lane A is the product of RNase U2 partial digestion. The enzymatic cleavage sites of the sequence position are indicated by the numbers at the left margin. The gel was a 15% polyacrylamide/7M urea (25 x 26 x 0.08 cm) and was run for 2.5 hours at 9mA.

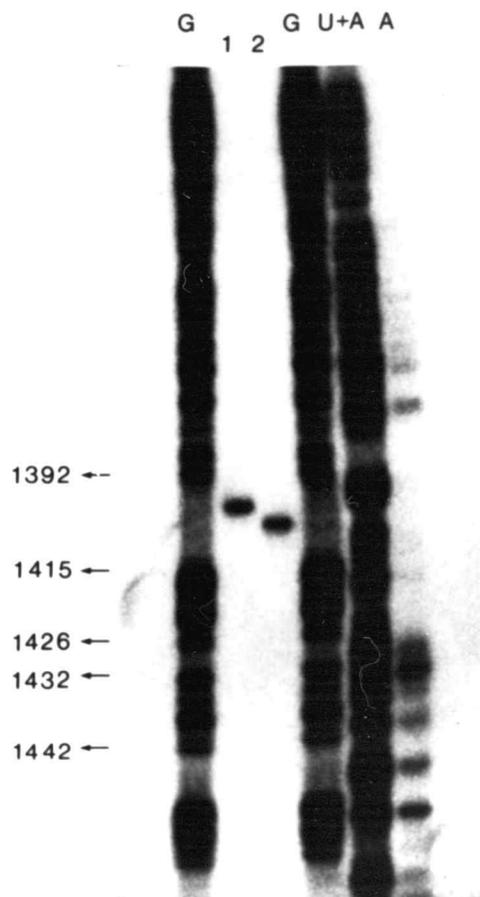


Figure A-2

Figure A-3. Autoradiograph of enzymatic RNA sequencing. The RNAs were 3'-end labeled with [^{32}P]pCp. Set 1 shows 16S rRNA and set 2 shows the large fragment generated using DNA probe 1396. The enzymes used were RNase Cl3, RNase U2, RNase Phy M, and RNase T1. The respective cleavage specificities are as follows: C, A, A+U, and G. Electrophoretic analysis was performed as described in Figure A-2.

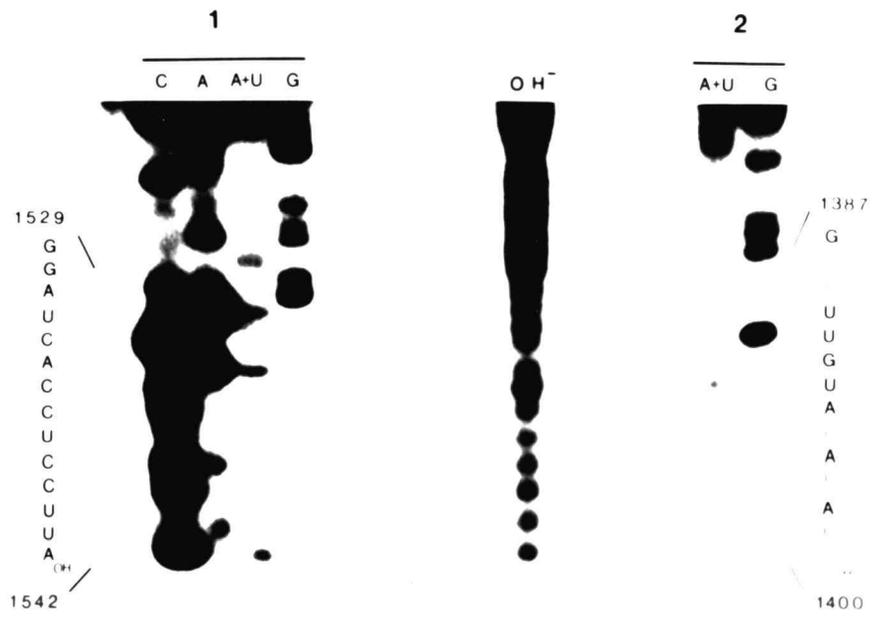


Figure A-3

Figure A-4. Scatchard plots of oligodeoxynucleotide binding to 16S rRNA and 30S ribosomal particle. Zones containing protected DNA similar to those shown in Figure 2-2 were excised from the gel and quantitated by liquid scintillation spectrometry in a Triton X-100 cocktail. The ratio of moles DNA bound per mole of 16S rRNA is plotted against the ratio of bound DNA to the concentration of nonprotected free DNA. The number of moles of DNA in the protected bands were calculated by using the specific activity of the respective probes. Figure A-4a represents the binding of DNA probe 1396 to normal 16S rRNA and Figure A-4b represents the binding of DNA probe del-1396 to the altered 16S rRNA (see Table 2-1 for the sequences of the DNA probes). Scatchard plots of the binding of DNA probes to 30S subunit are as follows: binding of DNA 520 to normal ribosome (A-4c) and to the altered ribosome (A-4d), and the binding of DNA 1493 to normal ribosome (A-4e) and to the altered ribosome (A-4f).

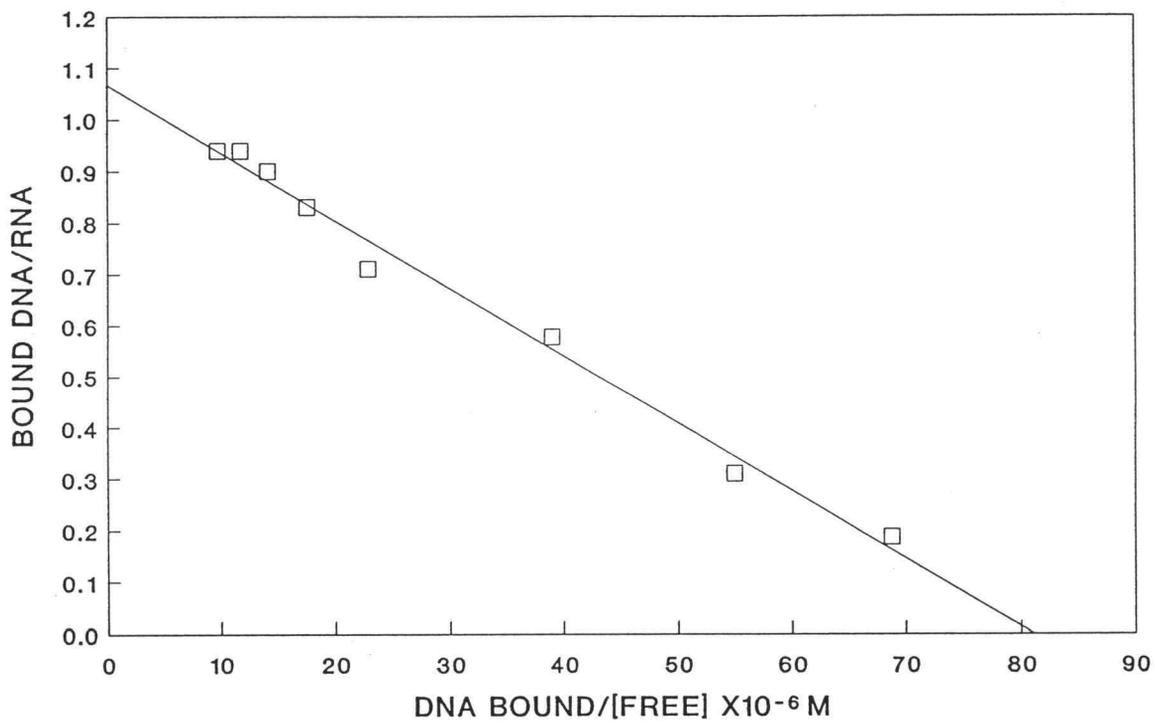


Figure A-4a

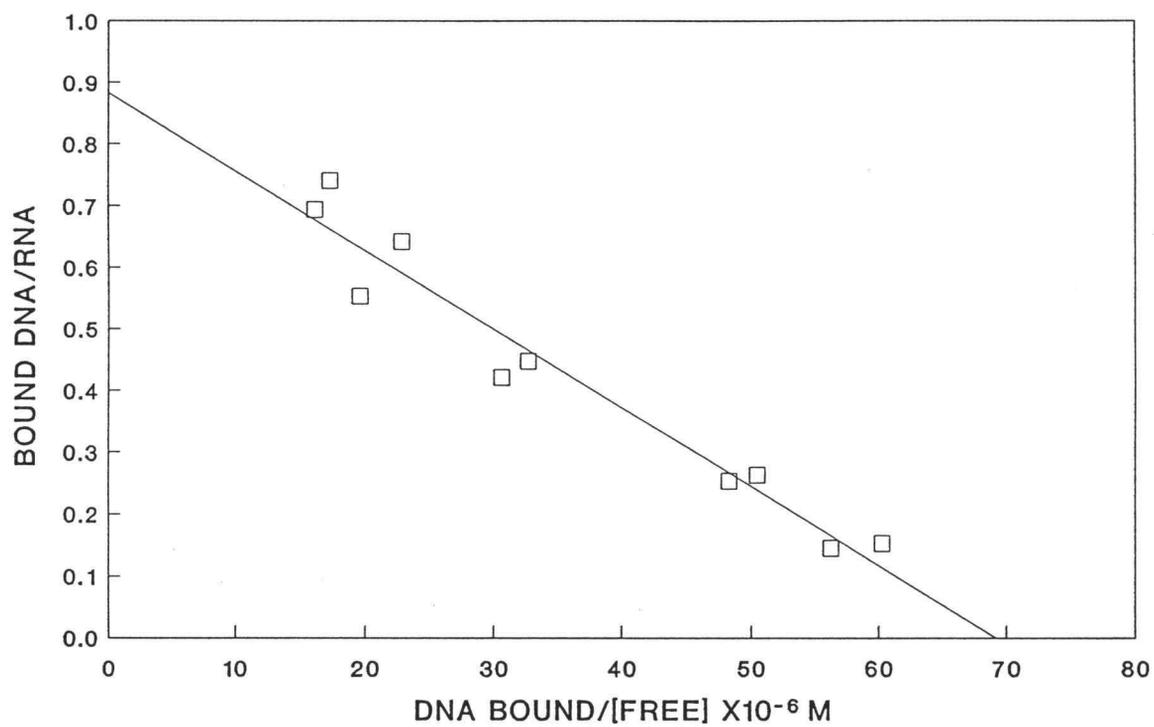


Figure A-4b

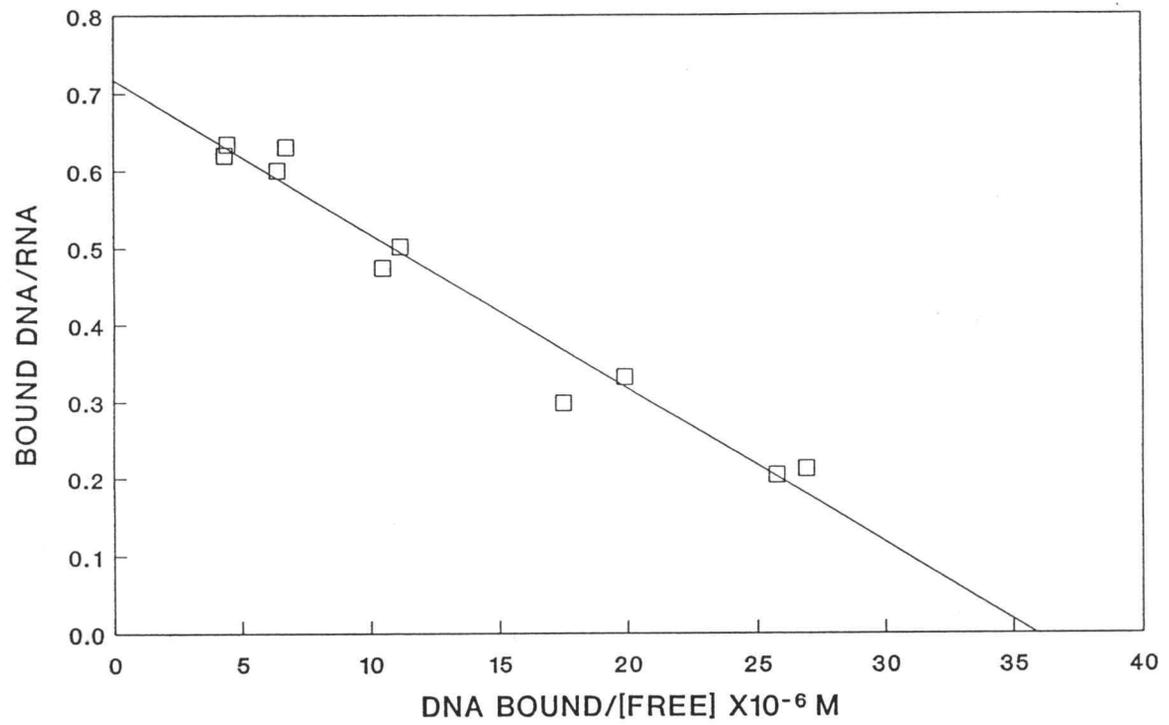


Figure A-4c

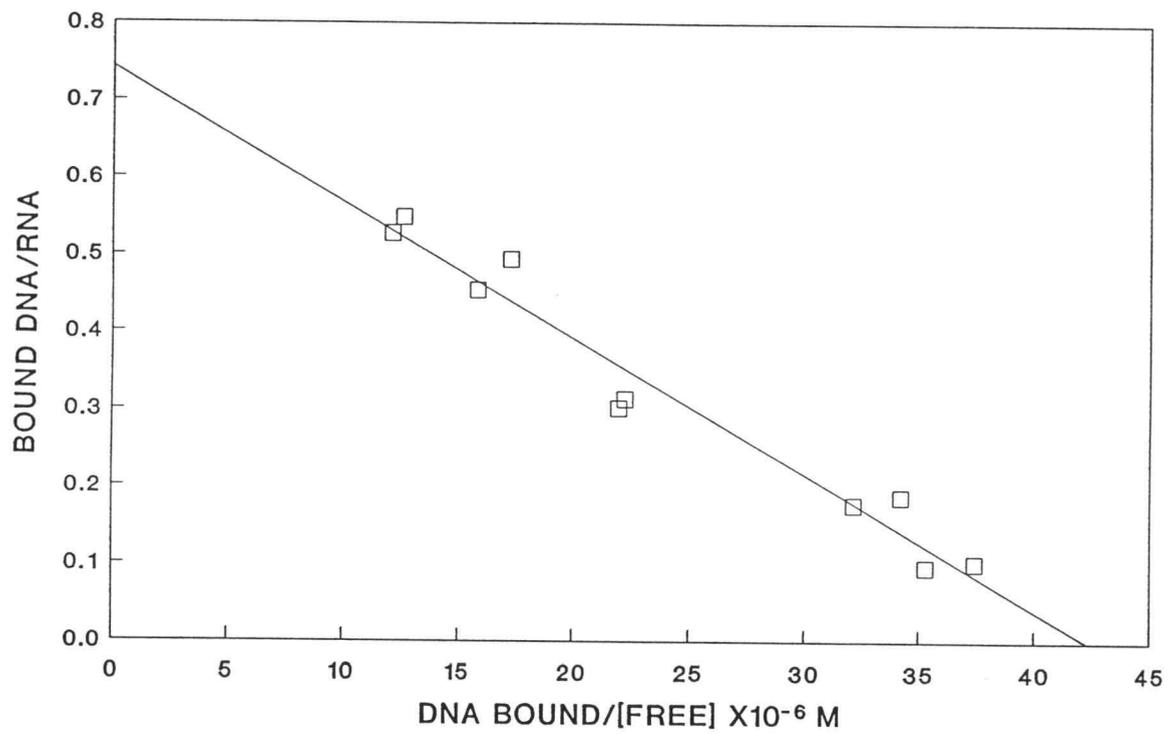


Figure A-4d

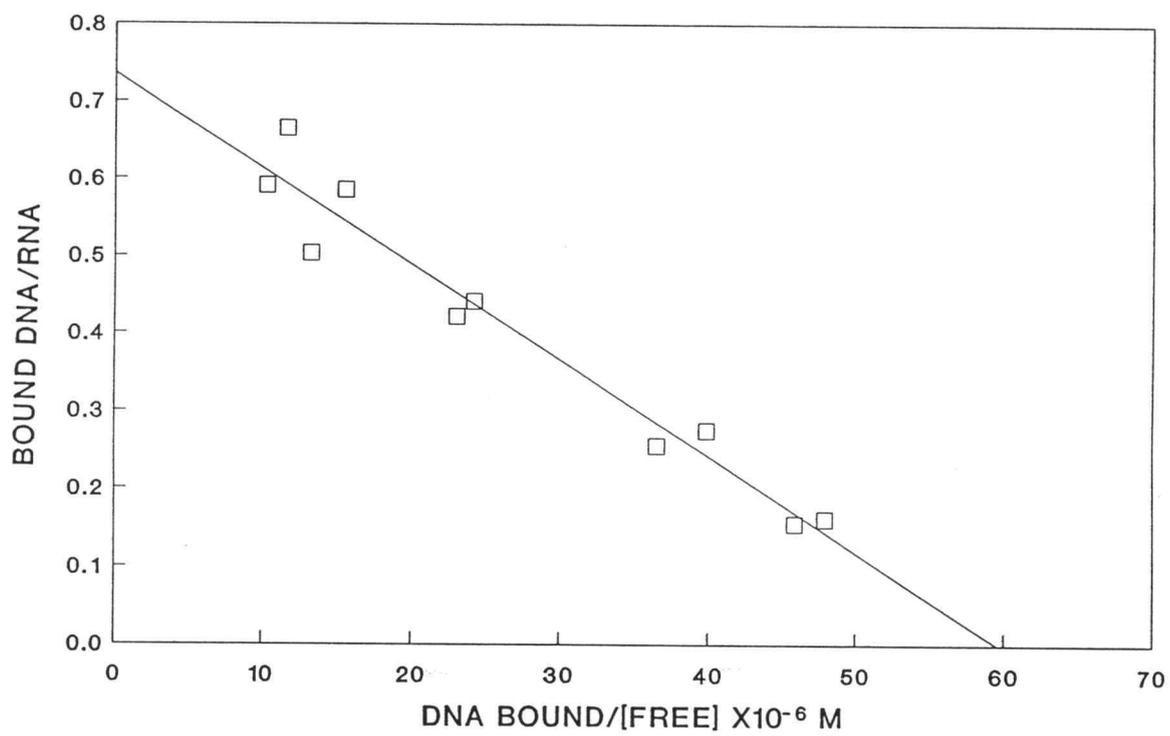


Figure A-4e

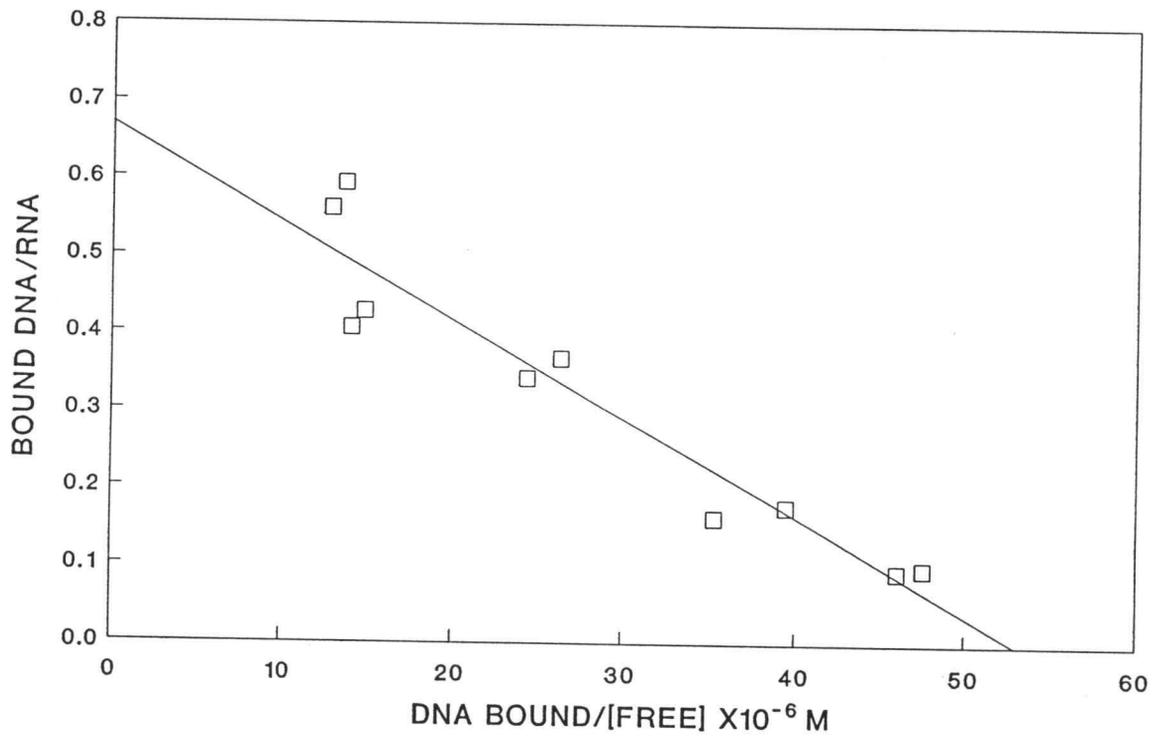


Figure A-4f

Figure A-5. Sucrose gradient sedimentation profile of 70S ribosomes. The gradient was 15-30% in gradient buffer (10 mM Tris-base, 50 mM KCl, 0.3 mM MgCl₂, pH 7.6). Sedimentation is from right to left showing the regions of 50S subunits, 30S subunits, and tRNAs. Centrifugation was performed at 43,000 xg, 4 °C for 16 hours in a Sorvall AH627 rotor.

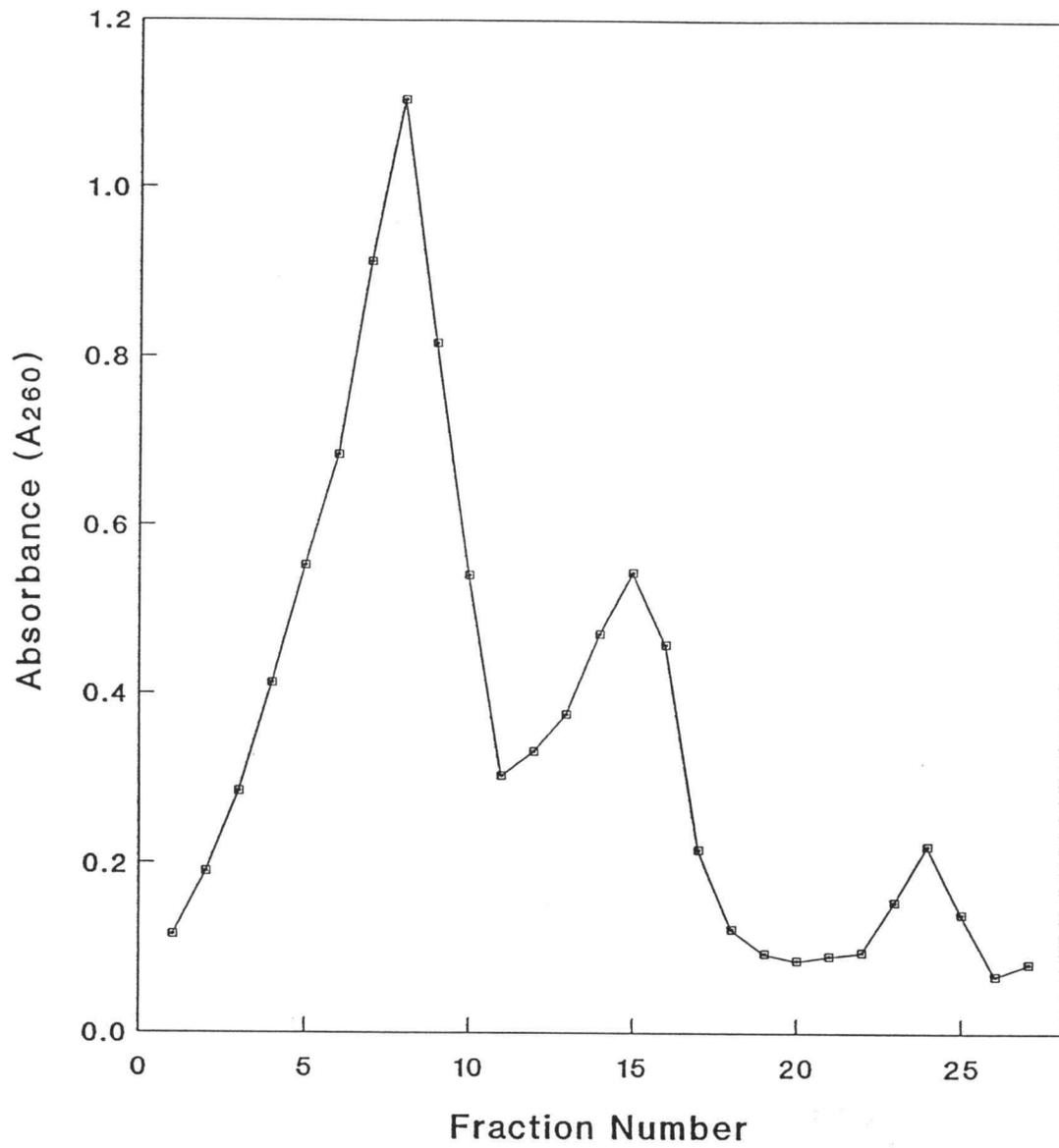


Figure A-5

Figure A-6. Sedimentation profile of total RNA extract from *E.coli* in 15 to 30% sucrose gradient in TSM buffer (10 mM Tris-HCl, 10 mM MgCl₂, 0.3 mM succinic acid, pH 8). Sedimentation was from right to left showing 23S rRNA, 16S rRNA, and small RNAs respectively. The separation was performed with a Sorvall AH627 rotor at 24,000 rpm for 21 hours at 4 °C.

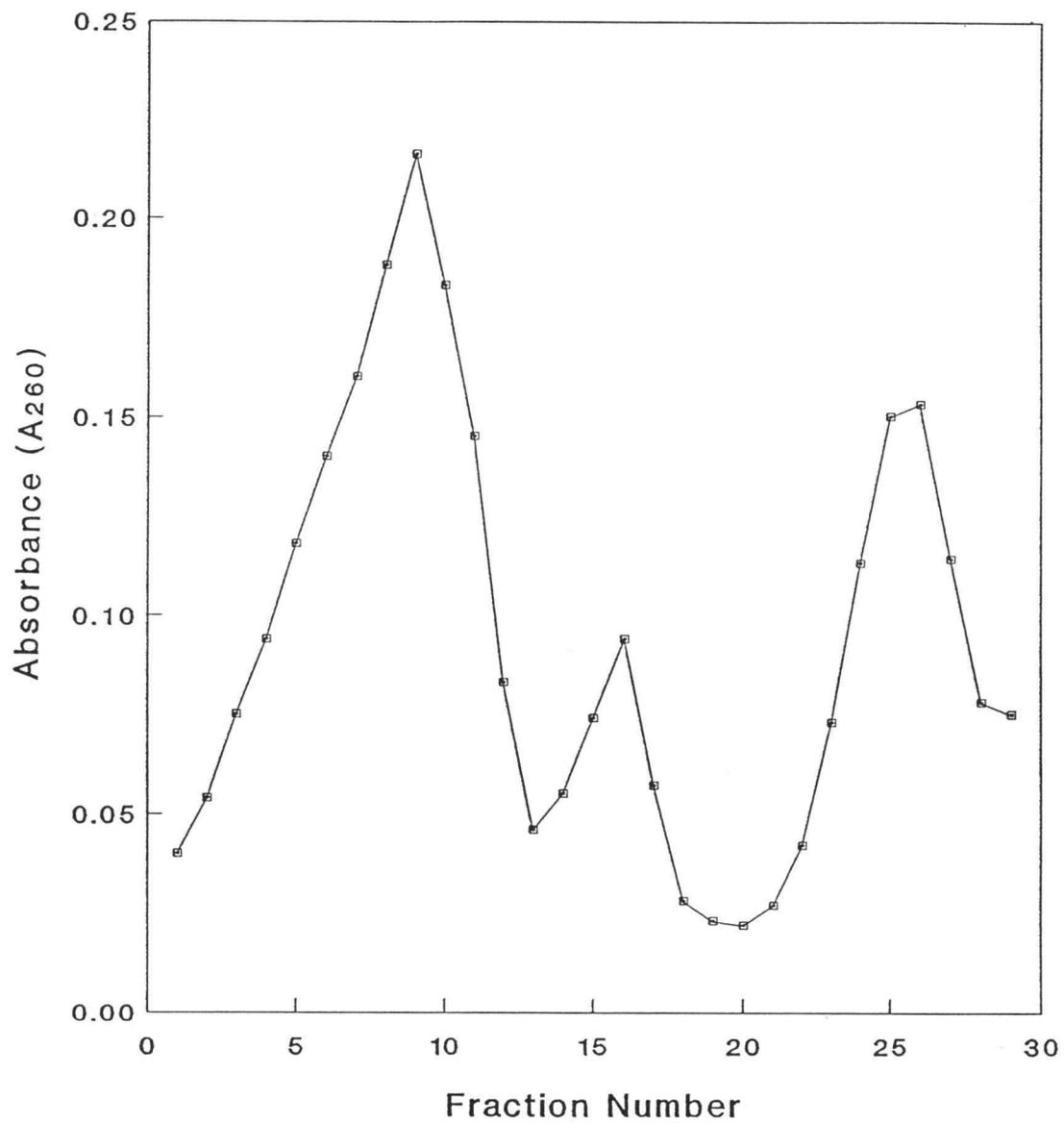


Figure A-6